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# NOTE ON A POSSIBLE SOURCE OF ERROR IN THE BELL-DOISY METHOD FOR THE DETERMINATION OF PHOSPHATES IN BLOOD PLASMA.

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Although but little more than a year has elapsed since Bell and Doisy<sup>1</sup> published a description of their method for the colorimetric determination of phosphates in urine and blood this technique has gained extensive use by investigators occupied with experimental work on the chemistry of blood.

In this laboratory an experience of 9 months, during which time we have had occasion to make about 400 determinations, has convinced us of the reliability of this procedure for the determination of inorganic phosphates in serums. When, however, we attempted to apply the method to plasmas, both citrated and oxalated, anomalous results were obtained. It was found that certain specimens of plasma gave no color when treated with the various reagents used in this method, in other cases the color obtained was much fainter than experience had led us to expect.

An investigation of the possible cause or causes of this phenomenon has led us to believe that this failure of color development is due to the presence of an excess of oxalate or citrate in the plasma.<sup>2</sup>

<sup>1</sup> Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

<sup>2</sup> This source of error has probably been recognized in part at least by other workers. Hess and Gutman (Hess, A. F., and Gutman, M. B., *J. Am. Med. Assn.*, 1922, lxxviii, 29) state that "it is important to use a minimum quantity of oxalate" but give no reason for this advice; while Myers and Shevsky (Myers, B. A., and Shevsky, M. C., *J. Lab. and Clin. Med.*, 1921, vii, 176) note that in certain specimens of rabbit plasma and in one specimen of human plasma no color could be obtained.

As it is frequently necessary to determine phosphates in plasma, we have carried out a series of observations on the effects produced by the addition of various amounts of sodium citrate and potassium oxalate to human and to horse blood, the results of which are collected in Table I.

TABLE I.

*The Inorganic Phosphate of Plasma Determined after the Addition to Blood of Various Amounts of Sodium Citrate and Potassium Oxalate.*

Subject.	Sodium citrate per cc. blood.	Potassium oxalate per cc. blood.	Inorganic phosphate of plasma per 100 cc.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Horse.	0.0		2.6
"	0.5		2.6
"	1.0		2.6
"	2.0		1.85
"	2.5		1.25
"	5.0		1.25
"	10.0		Too faint to read.
"	15.0		Colorless.
"	25.0		"
"		0.5	2.5
"		1.5	2.6
"		2.0	1.85
"		5.0	1.25
"		10.0	Too faint to read.
"		15.0	Colorless.
"		25.0	"
Man.	1.0		2.5
"	1.5		2.2
"	3.0		2.0
"		0.5	2.5
"		1.0	2.5
"		3.0	2.0

From the results presented in Table I, it is evident that in order to obtain correct results in the determination of inorganic phosphate in plasma by the Bell and Doisy method it is necessary to restrict the amount of anticoagulant added to such an extent as greatly to impair its efficiency. Potassium oxalate added to blood in the concentration of 10 mg. and sodium citrate in the concentration of 15 mg. per 10 cc. of blood, will retard coagula-

tion for but a limited time. Furthermore it is not always possible either with animals or with human subjects invariably to secure the quantity of blood planned for, a fact which makes it difficult to decide in advance the exact amount of anticoagulant to be added to the receiving vessel. Myers and Shevky<sup>3</sup> have recommended the use of amounts of molybdic acid and hydroquinone in excess of those specified by Bell and Doisy, as by means of this modification they were able to produce color in some of the trichloroacetic acid filtrates, which, when treated according to the original method gave no coloration. We have repeated the experiments of Myers and Shevky on this point and are able to corroborate the recommendation of these investigators regarding the desirability of using additional quantities of molybdic acid reagent and hydroquinone.

For work with horse or human (adult) plasma we have used 10 cc. of trichloroacetic acid filtrate (dilution 1:10), 2 cc. of molybdate reagent, and 2 cc. of hydroquinone solution of double strength (40 gm. per 1,000 cc.). With horse plasma, either citrated or oxalated, we have been able by this procedure to obtain figures identical with those given for serum with plasmas containing as high as 20 mg. of sodium citrate or 25 mg. of potassium oxalate per 10 cc. of blood. The presence of amounts of anticoagulant greater than this invariably gave values for inorganic phosphate lower than those obtained on the serum of the same animal.

From these results it would seem desirable whenever possible to make determinations of inorganic phosphates only on serum, and where plasma must be used to restrict the amount of oxalate or citrate, and increase the quantities of molybdic acid and hydroquinone as described above.

<sup>3</sup> Myers, B. A., and Shevky, M. C., *J. Lab. and Clin. Med.*, 1921, vii, 176.



# CHEMICAL CHANGES OF THE BLOOD UNDER THE INFLUENCE OF DRUGS.

## I. ETHER.

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### INTRODUCTION.

Ether glycosuria was shown by Seelig (1) to be associated with hyperglycemia. The mechanism of ether hyperglycemia has been studied by Keeton and Ross (2) who found about 100 per cent increase of blood sugar in normal dogs after they had been under ether for 2 hours. McGuigan (3) and many others corroborate this increase in blood sugar under ether anesthesia. It has been shown by Morriss (4) that ether anesthesia results in a lowering of the carbon dioxide-combining power of the blood plasma of dogs. Ross (5) found that the amount of decrease of amino-acids in dogs due to ether anesthesia follows the initial amino-acid content of the blood. He obtained no decrease when the amino-acid content of the blood was at the normal level, but when the amino-acid content was doubled by meat feeding he obtained a decrease of 9.2 per cent from this level. Bloor (6) found that ether anesthesia produced, in fasting dogs, a definite rise in the blood fat. In most cases investigation has been made of the change of a single constituent in each animal.

The present investigation is an attempt to correlate the changes in the blood constituents, under the influence of ether anesthesia. It would seem probable that the total changes in the more important blood constituents, in addition to clarifying metabolic changes, would furnish data for a more comprehensive analysis of the nature of anesthesia.



*Methods.*

Healthy and well nourished dogs were selected and placed on a mixed diet for several days before and throughout the experiment. The diet consisted of 200 gm. of lean beef heart, 100 gm. of cracker crumbs, 10 gm. of lard, and 10 gm. of bone ash. The dogs were fed late in the afternoon. Control samples (Column A in Table I) were drawn from the heart the next morning just before placing the dog under ether for 2 hours; a second sample was drawn just before removing the ether (Column B); and in a few cases a third sample (Column C) was taken on the following morning. The samples were drawn from the heart, and were usually about 20 to 30 cc. in volume. This is too small a volume, when medium sized dogs are used, to cause any of the effects due to hemorrhage. The dogs soon became accustomed to the method of drawing blood, and submitted without excitement.

The depth of the anesthesia was regulated by the corneal reflex just failing to be given.

In this study determinations were made on the same sample of blood—of sugar, hydrogen ion concentration, carbon dioxide-combining capacity of the plasma, creatinine, total non-protein nitrogen, urea nitrogen, total lipoids, cholesterol, and lecithin. The blood sugar was determined by the use of the second method of Folin and Wu (7).

The method and apparatus described by Clark (8) were used to determine the hydrogen ion concentration which we express as the pH. A saturated potassium chloride-calomel cell was made up every month and tested at frequent intervals against a buffer solution of potassium di-acid phosphate and sodium hydroxide, with a pH of 7.4. Several experiments were made in the beginning of the investigation on the relative value of the Clark (8) and McClendon and Magoon (9) electrodes. The results obtained showed a variation in pH of the same samples of blood of four or five hundredths, if the readings were taken within 10 or 15 minutes from the time of the drawing of the blood. These findings taken into consideration with the fact that the McClendon vessel is more difficult to manipulate determined our use of the Clark vessel in these experiments. Some work was also done on the use of whole blood and blood plasma. It was found that the whole

blood and the plasma—obtained by centrifuging the oxalated blood in a closed tube—gave results which did not differ more than 0.05. We therefore used the plasma because with it the hydrogen electrode vessels could better be kept clean.

The carbon dioxide-combining capacity of the blood plasma was determined by the method of Van Slyke (10); the creatinine by the method of Folin and Wu (11); the urea nitrogen by the method of Van Slyke and Cullen (12); total lipoids (13), cholesterol (14), and lecithin (15), according to Bloor with a slight modification. It was found both more convenient and more accurate to saponify both the standard oleic acid and the sample of blood lipoids in a Florence flask, dissolve in 5 cc. of ether-alcohol mixture, and add the water to this solution, thus avoiding any transfer of the solution with a possible loss and also securing the same color in both due to a slight darkening of the sodium ethylate. The non-protein nitrogen was determined by the method of Folin and Wu (11). A Kober nephelometer-colorimeter was used throughout.

#### EXPERIMENTAL DATA.

Table I gives the analytical results obtained.

In Table I, it is shown that on an average the carbon dioxide capacity of the blood plasma decreased from 45.7 volumes per cent to 28.8, or a change of 16.9 volumes per cent which is a relative decrease of 36.9 per cent in 2 hours under ether. The carbon dioxide had returned to normal the next morning.

The normal blood sugar was found to be 0.103 per cent and to be increased to 0.20 per cent after 2 hours under ether or a relative increase of 94 per cent. It was still 22.3 per cent above normal the next morning.

The non-protein nitrogen was at first increased slightly and then decreased. However, there are too few analyses to make a satisfactory average.

The urea at first increased and this higher level was maintained for at least 24 hours.

The creatinine was about 1.5 mg. per 100 cc. before putting the dogs under ether and this was increased 16.2 per cent in 2 hours, but had returned to normal the next morning.

TABLE I—*Effect of Ether Anesthesia on the*

Dog No.	Experiment No.	Date.	CO <sub>2</sub>			Sugar.			Non-protein nitrogen per 100 cc.			Urea per 100 cc.		
			A	B	C	A	B	C	A	B	C	A	B	C
		1921	vol. per cent	vol. per cent	vol. per cent	per cent	per cent	per cent	mg.	mg.	mg.	mg.	mg.	mg.
I	1	Sept. 6	48	22		0.105	0.167		31.2	42.0				
I	4	" 13	38	29		0.087	0.284		21.0	23.0				
II	2	" 7	47	31		0.108	0.296							
II	5	" 14	47	31		0.097	0.324							
II	6	" 26	48	33		0.120	0.278							
II	9	Oct. 4	52	35	46	0.122	0.226	0.141	34.4			19.6		
II	19	Nov. 1	47	29		0.092	0.174		34.4	23.8		17.6		
III	3	Sept. 9	28	18		0.095	0.188		36.4	37.1				
IV	8	" 30	47	28	47	0.095	0.308	0.153	38.5	42.5				
IV	10	Oct. 5	48	28		0.102	0.240	0.143						
VI	7	Sept. 27	47	26		0.108	0.148							
VIII	13	Oct. 19	47	27	47	0.082	0.095	0.079	26.6	26.6	25.2	19.6	14.9	22.4
VIII	14	" 21	47	34	50	0.081	0.115	0.100	22.4	25.2				
VIII	15	" 25	54	27	46	0.093	0.121	0.093	21.0	33.6	19.6			
VIII	16	" 29	46	26	45	0.098	0.174	0.118	24.6	22.4	21.0	13.9	18.6	18.9
VIII	20	Nov. 7	45	36	42	0.118	0.133	0.102	21.0	26.6	25.3	18.9	25.1	17.7
VIII	21	" 8	42	30		0.102	0.131		25.3	32.2		17.7	20.7	
Averages.....			45.7	28.8	46.1	0.103	0.200	0.126	28.0	29.5	22.7	17.9	19.4	19.6
Change, per cent..				-36.9	+0.87		+94.0	+22.3		+5.0	-18.9		+8.15	+9.44

Column A contains the readings taken for a normal dog; Column B, normal dog, 2 hours after

The normal total fat was 0.73 per cent and this showed a drop of 11.7 per cent and then an after rise in 24 hours of 26.7 per cent above normal.

The normal cholesterol of 0.16 per cent showed very little change, either during the narcosis or after the removal of the ether. The lecithins also changed very little.

The normal pH was found to be 7.38 and this was lowered 4.4 per cent to 7.06 in 2 hours under the influence of ether. It returned to normal in the next 24 hours.

#### DISCUSSION AND SUMMARY.

A full discussion of these results will be postponed until the completion of the investigation of other drugs now under way.

*Blood Constituents of the Dog.*

Creatinine per 100 cc.			Fat.			Cholesterol per 100 cc.			Lecithin.			pH		
A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
mg.	mg.	mg.	per cent	per cent	per cent	mg.	mg.	mg.	per cent	per cent	per cent			
			0.714	0.667		0.186								
1.80	1.68		0.769	0.682										
1.71	2.36		0.895	0.741										
1.15	1.25		0.750	0.706										
1.87	2.64		0.500	0.550										
1.38			0.75	0.83	0.69	0.153			0.50			7.50	7.01	
1.41	1.55		0.71	0.69		0.156			0.51	0.49		7.41	7.01	
												7.50	7.11	7.48
			0.80	0.71	1.84	0.303								
1.41	1.70			0.75										
1.85	2.60	1.88	0.79	0.74	0.79	0.088	0.188	0.156	0.45			7.35	7.05	7.36
1.11	1.20		0.78	0.73	0.93	0.119	0.141	0.166	0.50	0.60	0.45	7.35	7.10	
1.46	1.33		0.75	0.76	0.77	0.165			0.62	0.43		7.25	7.02	7.30
1.59	1.22	1.41	0.72	0.76	0.71	0.111	0.147	0.147	0.44	0.35		7.35	7.05	7.38
1.41	1.34	1.02	0.71	0.75	0.72	0.147	0.156	0.148				7.38	7.01	7.27
1.02	1.66		0.72	0.76		0.148	0.147		0.25	0.28		7.27	7.03	
1.47	1.71	1.43	0.726	0.641	0.92	0.157	0.156	0.152	0.47	0.43	0.45	7.38	7.06	7.38
	+16.2	-2.7		-11.7	+26.7		-0.5	-3.1		-8.3	-4.2		-4.4	-0.3

starting ether; and Column C, normal dog, 24 hours after Column A.

in this laboratory, in order that a better perspective may be had. Mere statements of fact, without attempt at explanation at this time would seem advisable. The results agree in general with the composite findings of the various individual analyses. Judging from the results of this investigation alone we might conclude:

1. The blood sugar content during ether, also the hydrogen ion value, is raised and the carbon dioxide capacity of the blood plasma decreased (confirmatory of Morriss (4) and of Carter (16)).

2. The cause of the rise in the sugar may be related to: (a) The increase in the hydrogen ion which corresponds to a decrease

in the pH; and (b) the decrease in the carbon dioxide capacity of the blood plasma.

3. The fat decreased during the ether narcosis and then showed an after rise above normal.

4. The cholesterol and lecithin change very little.

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## STUDIES IN CREATINE AND CREATININE METABOLISM.

### IV. ON THE QUESTION OF THE OCCURRENCE OF CREATININE AND CREATINE IN BLOOD.

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The very rapid advances in analytical technique applied to biological tissues and fluids made during the past 10 years have resulted in a rather anomolous situation. We are constantly determining substances whose existence in the tissues or fluids analyzed has never been proved, and on the basis of a single, non-specific color reaction reports are made of the quantity of a substance in a given tissue or fluid, although none of this substance has ever been separated as such from the material analyzed. The modern color reactions are very attractive playthings, but the facility with which they can be employed should not lead to neglect of the more fundamental work of seeking definitely to prove exactly what these color reactions may signify.

It is not intended, in this connection, to decry the use of modern analytical technique for blood and tissues—such studies should be pursued as far as they seem to promise results of value from any angle, but we should keep in mind the necessity of continued investigation of the tissues and fluids of the organism from the qualitative standpoint.

Over a year ago one of us encountered very anomolous results during an investigation of the question of the probable interference of creatinine when using the picrate method for determination of blood sugar. These results, some of which are cited below, were so at variance with known reactions of creatinine that they appeared to demonstrate that in some bloods at any rate, creatinine could not be present in anything like the quantity indicated by the colorimetric method of Folin for determining this substance. We were therefore led to take up a more detailed study of this question, and to include a study of the creatine content of blood.

The question of the existence of creatinine in blood has already been raised by others. Hunter and Campbell (1) in 1917 reported a careful study of the question, based chiefly upon comparing the rate of color development of creatinine in alkaline picrate solution with color development in blood filtrates under corresponding conditions. Curves were constructed which showed the velocity of color development in this reaction, the amount of color being plotted against the time. With pure creatinine solutions it was found that the curve was typical and that solutions of different concentrations show proportionate color production. Various blood filtrates were compared with creatinine solutions, with the result that Hunter and Campbell concluded that Folin's picric acid method can be used to determine the creatinine in the plasma with accuracy, but that there is an additional substance in whole blood (present in the corpuscles) which increases the color of the reaction due to creatinine by about 50 per cent, while autoclaved filtrates from blood and plasma contain a still greater amount of a substance, not creatinine, which contributes 50 to 75 per cent of the color usually attributed to creatinine.

These authors did not question the presence of a certain amount of creatinine in the blood but they suggested that a considerable part of the color was probably not due to creatinine.

Greenwald and McGuire (2) have also studied certain factors bearing on the question of the true creatinine and creatine content of the blood. These authors did not draw definite conclusions as to the probable occurrence of these substances in blood, but a reading of their paper convinces one that they held serious doubts as to the true nature of the "creatinine" in blood. Their attempts to isolate creatinine from blood failed in every instance, and their final opinion is quite suggestive of suspicion of the whole subject. Thus they state:

"For an investigation of normal creatine and creatinine metabolism, the methods are probably not satisfactory. Until it can be shown that the chromogenic substance is really creatinine, investigations in this field would seem to be of doubtful value."

The available evidence that creatinine exists in blood may be briefly summarized as follows: (1) Creatinine occurs in the urine, hence it is probably present in the blood and (2) the rate of color

development in alkaline picrate solutions due to the blood component, closely approximates that found for pure creatinine solutions. Obviously neither of these arguments for the presence of creatinine in blood, in the amounts indicated by present quantitative methods is at all conclusive. Concerning the first argument it may be pointed out that creatinine may be produced by the kidney, or the kidney may be able to concentrate this substance from dilutions in the blood very much greater than is commonly assumed. The point established by Hunter and Campbell also fails to demonstrate the existence of creatinine in blood. An unknown blood constituent might duplicate the rate of color production, and the possible presence of catalysts affecting the reaction in blood filtrates, as suggested by Greenwald (2), must be taken into consideration. We are therefore of the opinion that no results so far available offer definite evidence of the existence of creatinine in blood.

Our method of studying the question has developed along several lines. We have applied certain reactions to both blood filtrates and pure creatinine solutions, and to creatinine added to blood, and compared the relative behavior of true creatinine and of the blood creatinine, both before and after conversion of possible creatine into creatinine. We have also made studies based upon the use of adsorptive reagents which will remove true creatinine from pure solution or from blood filtrates.

In most of the creatinine determinations we have employed the original picric acid method of Folin (3), in which the blood is diluted to five times its volume with saturated picric acid, and the total solution saturated with picric acid. For carrying out this saturation with dry picric acid we have placed the mixtures in a shaking machine for from 5 to 10 minutes.

Our studies in connection with this method have shown that not more than 4 or 5 mg. of creatinine added to 100 cc. of blood can be recovered quantitatively by this technique. Doubling the volume of dilution permits the satisfactory recovery of the larger quantities of creatinine. We have also employed precipitation by means of heat coagulation, trichloroacetic acid, and tungstic acid (Folin and Wu, 4), followed by saturation of the filtrate (after exact neutralization in the case of the trichloroacetic acid precipitation) with picric acid. All of the picric acid employed has at least fulfilled



the requirements of purity indicated by Folin and Doisy (5) as necessary for creatinine determination in blood.

Results by the various methods of precipitation indicated that for normal bloods the various precipitants yield parallel figures, while for bloods high in color-reacting substance the Folin-Wu precipitation with tungstic acid usually yields much higher figures than does the picric acid precipitation. Heat coagulation filtrates show still higher figures (Table VI).

As mentioned earlier in this paper, our attention was first directed to the question of the occurrence of creatinine in blood by the results obtained by one of us in a study of the possible interference by creatinine in the picrate method for blood sugar determination. It was found that creatinine added to blood caused an increase in the apparent blood sugar equivalent, in terms of glucose, to about three times the quantity of creatinine added, provided that this was more than about 3 mg. It was also found that purified bone-black, when used in quantities of about 1.5 gm. for the 25 cc. of blood picrate mixture, after a few minutes shaking would remove up to 25 mg. of creatinine per 100 cc. of blood. When the bone-black procedure was applied to bloods containing high non-protein nitrogen it was found that while such bloods very commonly showed a marked drop in sugar content after treatment with bone-black, in many instances this drop bore no relationship to the creatinine content of the blood. Furthermore, some bloods which gave a high creatinine content by the regular method, showed no drop whatever in the sugar content when treated with bone-black.<sup>1</sup> A few typical figures in this connection are given in Table I.

An inspection of Table I shows that there is little relationship between the fall in the sugar content of the blood after treatment with bone-black and the creatinine content of the blood. The results obtained with Samples 7, 12, and 13 render it very improbable, or indeed impossible, that creatinine should have been

<sup>1</sup> It may be noted here that most bloods showing a high non-protein nitrogen content show a marked drop in sugar content as determined by the Folin-Wu method after treatment with bone-black, and a still greater drop with Lloyd's reagent. The Folin-Wu procedure cannot be influenced by creatinine, or any other known constituent of the blood except glucose. It is hoped that this work in connection with blood sugar determination can be reported upon in the near future.

present in these samples in the quantities indicated by the recognized method for the determination of this substance. Thus Sample 12, with a creatinine content of 8 mg. per 100 cc. which should be equivalent to about 24 mg. of glucose in the picrate method, shows no appreciable drop after treatment with bone-black, which removes pure creatinine.

TABLE I.

*The Effect of Bone-Black Treatment on Blood Sugar Determinations in Relation to Blood Creatinine.*

Sample No.	Sugar without treatment with bone-black per 100 cc. of blood.	Sugar after treatment with bone-black per 100 cc. of blood.	Difference per 100 cc. of blood.	Creatinine per 100 cc. of blood.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	96	88	8	1.2
2	112	93	19	1.2
3	150	132	18	2.0
4	280	220	60	6.0
5	186	179	7	4.2
6	125	110	15	3.1
7	116	112	4	5.0
8*	119	101	18	1.6
9	124	111	13	1.7
10	137	122	15	1.4
11	108	81	27	1.0
12	107	104	3	8.3
13	142	142	0	4.6
14	117	118	1	1.4
15	120	104	16	1.4

\* Figures on Samples 8 to 15 were obtained by Dr. Gertrude F. McCann in the laboratory of Dr. Frederick M. Allen. We are indebted to Dr. McCann and Dr. Allen for permission to use this material.

Obviously such results as are reported in Table I cannot do more than show that any particular sample of blood does not contain the creatinine ascribed to it by another and more direct method of determination. Nevertheless, such results do throw doubt upon the validity of the general assumption that the picric acid reaction, as applied for creatinine, really determines that compound in blood.

Hence we were led to study the question further. A peculiarity of picric acid filtrates from blood which we have repeatedly noticed, and which has probably impressed others, is that such filtrates are very frequently definitely darker in color than pure picric acid solutions, without addition of any alkali. Thus a colorimetric reading often showed such filtrates to have twice as much color as a saturated aqueous solution of picric acid. This observation indicated a tendency on the part of some blood constituent to reduce picric acid in acid solution. Thus we were led to try the effect on color development in picrate solution when sodium carbonate is used as the alkali instead of sodium hydroxide, and to compare the results thus obtained with those yielded by pure creatinine under similar conditions. As an arbitrary standard for color comparison in this connection we employed a pure saturated picric acid solution, adding 0.5 cc. of 20 per cent sodium carbonate to each 10 cc. of solution, and making the readings after 10 minutes. When sodium carbonate is added to picric acid the depth of color in the solution increases slightly. If creatinine is present, in such quantities as we assume in blood filtrates, this increase in color is slightly greater, but is scarcely more than detectable, and is not definitely proportional to the creatinine present. In fact, increasing the creatinine five times yields a scarcely detectable increase in color when carbonate is used as the alkali. When carbonate is added to the picric acid filtrate from blood, there is a marked increase in color, and this color is far greater than could possibly be due to the creatinine content of the blood, as indicated by the regular determination. In other words, there is in blood a substance other than creatinine which reduces picric acid in the presence of sodium carbonate. It is fair to assume that this substance contributes to the reaction when hydroxide is employed as the alkali. Whether the reaction obtained with hydroxide is wholly due to the same substance which reacts (incompletely?) with carbonate cannot be answered. The increase of color in the blood filtrates with carbonate is usually particularly great in bloods which showed an abnormally high "creatinine" content, but in many other cases the increase due to carbonate is not proportional to that obtained with hydroxide. This may be due, as it is in the case of creatinine, to the fact that the reaction given by the blood compound is very incomplete

in presence of carbonate. We are inclined to believe that this explanation is correct, and that the "creatinine" in blood differs (among other particulars) from creatinine in that it reacts more strongly with picric acid in presence of carbonate. The evidence in this connection is, we admit, inconclusive beyond showing that results with hydroxide cannot be assumed to represent the true creatinine content of the blood.

More definite evidence that the "creatinine" of blood is not creatinine is furnished by experiments based upon the fact that creatinine is destroyed by heating in alkaline solution. We have found that if solutions containing up to 4.5 mg. actual concentration of creatinine per 100 cc. (corresponding to 22.5 mg. of creatinine per 100 cc. of blood where a 1 to 5 dilution has been employed) to which have been added 2 per cent of sodium hydroxide, are heated for 1 hour, the creatinine has been completely destroyed so far as its power of yielding color in alkaline picrate solution is concerned. When filtrates from normal blood precipitated by tungstic acid according to Folin and Wu (4) are heated with alkali under similar conditions, the blood "creatinine" remains practically unchanged. When similar filtrates from blood to which creatinine has been added were treated in a similar way, the added creatinine was lost, while the blood creatinine was unchanged. We will cite two examples in this connection. The tungstic acid filtrate from a sample of beef blood showed a "creatinine" content for the original blood of 1.94 mg. per 100 cc. 20 cc. of this filtrate were treated with 5 cc. of 10 per cent sodium hydroxide, and the tube was heated in the water bath for 1 hour. The solution was then neutralized exactly with hydrochloric acid, saturated with picric acid, and the creatinine determined as usual. The color obtained corresponded to 1.82 mg. of creatinine per 100 cc. of blood, against the 1.94 mg. found without the treatment with alkali.

When 2 mg. of creatinine per 100 cc. were added to this same blood (and recovered quantitatively in the filtrate), the reading after heating with the alkali showed 2.0 mg. per 100 cc. against 1.9 mg. in the original blood, and 4.0 mg. in the blood with the added creatinine. In another blood sample the filtrate showed 1.96 mg. of creatinine before heating with alkali and 2.1 after such heating. Addition of creatinine to this same blood so that the

filtrate showed a total of 8.60 mg. was then made. After heating with alkali the "creatinine" content had fallen to 2.50 mg., against 1.96 mg. in the original blood. In other words, more than 6 mg. of added creatinine per 100 cc. of blood were destroyed, while the original blood "creatinine" was practically unaffected.

Additional experiments with alkali gave similar results, but they also led to the discovery that glucose heated under similar conditions with alkali may give rise to products which simulate creatinine in the picric acid reaction. If the glucose concentration in the original blood be not greater than 0.2 per cent there is no interference due to the split-products, but with higher glucose concentrations the split-products may replace part of the creatinine lost through the action of the alkali. The results cited above were obtained with bloods of low sugar concentration, and thus furnish quite direct evidence that blood "creatinine" is not creatinine. Our finding that high concentrations of glucose would interfere with the alkali study of the creatinine question on many bloods, led us to abandon this line of work until we had looked further for a method of attack less open to possible objection. The question of the occurrence of creatinine in blood is of such importance that definite conclusions with regard to it should not be based upon a line of study open to possible objection along any line.

Our next study was concerned with the question of the removal of creatinine, and of the creatinine-reacting substance, from solutions by means of kaolin.

Greenwald and McGuire (2) have reported that kaolin removes creatinine quantitatively from dilute solutions, and based a method for determination of creatinine and creatine in blood upon this fact. In their study Greenwald and McGuire employed blood filtrates obtained by heat coagulation of the blood proteins in presence of dilute acetic acid, and assumed, upon the basis of their results with pure creatinine, that the creatinine-reacting substance in blood was also removed by treatment with kaolin. They apparently failed to make creatinine determinations directly upon filtrates after treatment with kaolin. Had they not neglected this point, Greenwald and McGuire could hardly have escaped the conclusion that there is no creatinine in blood.

Using the heat coagulation filtrate under the conditions prescribed by Greenwald and McGuire, we have found that true

creatinine is removed from pure solution or from blood filtrates quantitatively up to amounts corresponding to about 4 mg. of creatinine per 100 cc. of original blood. With higher amounts of creatinine the removal by kaolin is not quantitative. Employing the heat coagulation filtrates, the removal of the creatinine-reacting substance of the blood by kaolin is irregular and uncertain, even when only small quantities of the substance are present. In Table II are cited typical results in this connection upon one species of blood (beef).

TABLE II.

*Showing Results of Kaolin Extraction of Filtrate Obtained by Heat Coagulation of Beef Blood, with and without Addition of Creatinine.*

Sample No.	Blood without added creatinine.*			Blood with added creatinine.			
	Before extraction with kaolin.	After extraction with kaolin.	Amount removed by kaolin	Before extraction with kaolin.	After extraction with kaolin.	Amount of added creatinine.	Total amount removed by kaolin.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	2.32	1.50	0.82	3.24	2.18	0.92	1.06
2	2.32	1.50	0.82	4.75	1.72	2.43	3.03
3	2.14	1.05	1.07	5.04	2.16	2.90	2.88
4	2.32	1.50	0.82	6.12	3.36	3.80	2.76
5	2.37	1.05	1.32	6.10	1.94	3.75	4.16
6	3.06	2.14	0.92	8.05	2.40	5.00	5.65
7	3.51	1.83	1.68	8.61	3.52	5.10	5.09
8	3.21	1.87	1.34	8.61	4.02	5.40	4.59

\* All figures are in terms of milligrams of creatinine per 100 cc. of blood.

It will be noted that the quantity of the blood "creatinine" extracted by the kaolin would average from about 30 to 50 per cent. The same results hold true for the blood of other species. These results may perhaps explain the very irregular creatinine values reported by Greenwald and McGuire, using their method, as compared with figures obtained for the same samples by the Folin method.

As a result of our work with kaolin upon the heat coagulation filtrates we soon gained the impression that the amount of the blood chromogenic substance removed by the kaolin from these filtrates depended upon variable factors, which could not be well

controlled. Hence we set out to find conditions yielding more constant results in connection with the use of kaolin.

Experiments showed that in solutions completely freed from protein, kaolin,<sup>2</sup> used in a quantity of 2 gm. for 25 cc. of solution will remove creatinine quantitatively from solutions containing up to about 0.7 mg. per 100 cc. actual concentration, which corresponds to 3.5 mg. of creatinine per 100 cc. of blood. The mixture with kaolin was shaken in a shaking machine for about 10 minutes. The solutions we employed were acid with picric, trichloroacetic, or hydrochloric acids. Such conditions are safest for extraction of creatinine. Pure creatinine is removed by kaolin from neutral solution, but the presence of even the minutest amount of alkali prevents this. Even calcium carbonate will interfere with removal of creatinine from a solution by means of kaolin.

Employing kaolin as above indicated upon blood filtrates obtained after a 1 to 5 dilution and saturation with picric acid as in the Folin method for creatinine determination, our results appear to show conclusively that normal beef, dog, or human bloods contain no creatinine within the limit of accuracy of the method as we employed it. Creatinine in pure picric acid solution, or added to blood, is practically quantitatively removed up to about 3.5 mg. per 100 cc. of blood, while the creatinine chromogenic substance in the blood is unaffected. A hundred or more analyses have been carried out along this line. In no instance has there been an appreciable amount of the chromogenic substance of normal blood removed by kaolin, nor have we ever failed to remove added creatinine satisfactorily by the use of kaolin. Typical results in connection with this study are given in Table III.

In connection with the results reported in Table III it should be remembered that creatinine in pure saturated picric acid in concentrations equal to those reported for the bloods, is removed so completely by the kaolin treatment that such filtrates show only

<sup>2</sup> We have employed several different samples of kaolin with identical results. Eimer and Amend's "Kaolin, Acid Washed" was the one we used chiefly. We have also used Eimer and Amend's "Kaolin" which we ourselves washed with hydrochloric acid. Dr. Greenwald kindly sent us a sample of the kaolin employed by him. This preparation gave similar results to the other products we used.

TABLE III.

*Showing Removal by Kaolin from Picric Acid Filtrates of Creatinine Added to Blood, and That the Chromogenic Substance Is Unaffected by Treatment with Kaolin.*

Source of blood.	Blood without added creatinine.*		Blood with added creatinine.			
	Before extraction with kaolin.	After extraction with kaolin.	Before extraction with kaolin.	After extraction with kaolin.	Amount of creatinine added.	Amount removed by kaolin.
	mg.	mg.	mg.	mg.	mg.	mg.
Beef.	1.86	1.80	2.91	2.01	1.05	0.90
"	1.50	1.35	2.40	1.45	0.90	0.95
"	1.29	1.33	2.24	1.29	0.95	0.95
Dog.	1.27	1.18	1.92	1.33	0.65	0.59
Beef.	1.29	1.33	3.45	1.50	2.16	1.95
"	1.86	1.80	4.45	2.26	2.59	2.19
"	1.29	1.33	4.05	1.56	2.76	2.49
"	1.90	1.96	5.04	2.16	3.14	2.88
"	1.50	1.35	4.70	1.78	3.20	2.92
"	1.86	1.80	5.25	2.00	3.39	3.25
Dog.	1.27	1.18	4.85	1.47	3.58	3.38
Beef	2.40	2.18	6.40	2.37	4.00	4.03
"	1.29	1.33	5.30	1.86	4.01	3.44
Human.	1.84	1.71	4.55	2.00	2.71	2.55
"	3.36	3.60	6.06	3.90	2.70	2.16
"	3.60	3.27	5.76	3.81	2.16	1.95
"	1.57	1.26				
"	1.00	1.15				
"	1.23	1.02				
"	1.96	2.12				
"	1.09	1.25				
"	1.44	1.18				
Beef.	2.28	2.28				
"	2.15	2.35				
"	2.34	2.22				
Dog.	1.21	1.23				
"	1.27	1.18				
"	1.88	1.82				

\* All figures are in terms of milligrams of creatinine per 100 cc. of blood.

about 0.35 mg. of creatinine, which is the amount usually obtained with a blank against a "0.5 mg. standard" creatinine solution.

The figures reported in Table III are, as stated above, typical



of the results obtained in more than a hundred analyses. Plasma yielded results similar to those obtained from whole blood. We have included experiments showing maximal removal of the blood "creatinine," as well as those in which removal of added creatinine was least satisfactory. It appears that the results reported in Table III furnish convincing evidence that the bloods studied do not contain creatinine, or at least do not contain this substance in excess of a few hundredths of a milligram per 100 cc. Exceptions to this statement might be taken, based upon occasional results where kaolin treatment lowers the creatinine value of the blood by a few tenths of a milligram per 100 cc. But conversely it may be noted that instances also occur where the chromogenic value of the blood is increased by a few tenths of a milligram after treatment with kaolin. The method of study employed has its limits of accuracy. The probable error for a large number of analyses would not, we believe, exceed about 0.05 mg. of creatinine per 100 cc. Our results as a whole indicate that the true creatinine content of blood cannot exceed this figure, and we feel that it must probably be well below it.

Having demonstrated the absence of creatinine in detectable amounts from normal blood, it was considered important to determine whether creatinine accumulates in the blood in conditions of renal insufficiency or of complete ablation of renal function. Results here should indicate whether the creatinine in the urine is secreted by a process of concentration from undetectable traces which occur in blood, or whether the kidney itself produces creatinine. In this connection we have studied bloods obtained from human cases where the kidney function was impaired, and also bloods obtained from dogs 42 to 72 hours after extirpation of the kidneys or after ligating the ureters.

The results in connection with the high bloods are not conclusive on the question of the existence of true creatinine in these bloods. Human bloods yielding figures up to about 4 mg. of creatinine per 100 cc. show no loss of the chromogenic substance after treatment with kaolin. Higher bloods show a loss, following treatment with kaolin, amounting to from 20 to 50 per cent, providing the initial dilution of these bloods is great enough so that the actual concentration of reacting substance is about the same as in normal bloods. Such filtrates may also lose a considerable

percentage of color-yielding power (70 to 80 per cent) after heating with alkali, as detailed earlier in this paper. Very similar results were obtained with the dog bloods after ablation of the kidney function.

We should be inclined to the view that true creatinine accumulates in the blood after impairment of the kidney function, were it not for the following considerations:

After the demonstration that creatinine does not occur in normal blood in detectable amounts, more definite evidence is needed to prove its accumulation in abnormal blood than one or two non-specific reactions. Failure of removal by kaolin or of destruction by alkali may well demonstrate that a compound is not creatinine, but reverse findings do not demonstrate that a substance is creatinine. We are especially cautious in subscribing to the view that creatinine accumulates in the blood under the special conditions above cited because of the failure of an isolation experiment to demonstrate the presence of this compound. By the use of Lloyd's reagent a technique was developed which permits isolation of minute amounts of creatinine from large volumes of solution. So far only a single isolation experiment has been carried out in this connection, upon blood obtained from a dog after ligation of the ureters. The result was wholly negative for the isolation of creatinine. Though the final solution showed colorimetrically a creatinine content of about 9 mg. in 5 cc. of solution, creatinine-zinc chloride failed to separate from the solution after standing 2 days. After the addition of 5 mg. of creatinine to this same solution the added creatinine was recovered almost exactly as the zinc salt within a period of 3 hours.

It is this result which makes us feel that more data are needed before any conclusion can be drawn concerning the true creatinine content of the "high creatinine" bloods. In the near future we shall report further data upon this problem together with full details of our isolation experiments. Studies on the muscle creatinine are also contemplated.

After our investigation of the creatinine content of the blood we were led to study the behavior of the blood "creatine" and to attempt to find out whether all or part of this represents true creatine. The study has been complicated by the lack of an adequate method for conversion of creatine into creatinine in the

concentration found in the blood, which can be relied upon not to yield decomposition or other products which seriously interfere with the process.

A review of the literature on methods of creatine determination in blood cannot but leave one with the impression that each investigator, using a new technique, and one seemingly accurate, is able to get figures quite different from those obtained by any previous method.

The original method for conversion of creatine into creatinine in blood filtrates was proposed by Folin (3) and consisted in heating the filtrates with picric acid. Wilson and Plass (6) and Hunter and Campbell (1) have raised the question of the accuracy of creatine determination by this procedure. Folin and Wu (4) subsequently recognized the fact that such a procedure led to high results for the "total creatinine" and suggested that traces of hydrogen sulfide were formed during such heating. Folin and Wu, however, did not seem to give much weight to their own suggestion, for their new method for blood creatine included autoclaving blood filtrates, and it is not apparent why picric acid filtrates should evolve hydrogen sulfide any more easily than should any other blood filtrate.

Our experiments in connection with the picric acid filtrates obtained from blood have shown that blood contains some substance other than creatine, which rapidly reacts with picric acid in hot solution to give a product yielding color on addition of alkali. After heating the picric acid filtrates from normal bloods in the water bath for 1 hour the "creatinine" value is usually doubled, and after 3 hours heating the value is about trebled. In a case of human nephritic blood we have obtained an increase in color-yielding power equivalent to 6 mg. of creatinine after 15 minutes heating of the picric acid filtrate in boiling water. The increases due to such heating are out of all proportion to possible conversion of pure creatine into creatinine under identical conditions. Similar results are not obtained if other acids (acetic, trichloroacetic, hydrochloric) are substituted for the picric acid. Hence the reaction is not a change in the blood constituent due to heating with dilute acid, but is a specific reaction between the blood compound and picric acid in hot solution. Apparently the compound giving rise to this reaction is not the same as that re-

sponsible for the creatine reaction of the blood filtrate when heated with acid in the absence of picric acid, for after treatment which will completely convert creatine into creatinine there is still an increase if the solutions are heated with picric acid prior to addition of alkali, but the increase in color is not as great as before treatment with acid, due probably to the conversion of the creatine. If filtrates from plasma instead of whole blood be heated after saturation with picric acid the curve of increase in color-yield after addition of alkali is similar to that for whole blood, but the actual increase is only about one-half as great (*cf.* also Wilson and Plass, 6). We have no clue as to the nature of the substance reacting in hot picric acid solution to yield the increased color. Neither glucose nor other known constituents of the blood can apparently be responsible for the reaction. The substance is partially, but not completely, removed by treatment with kaolin.

Although blood contains this substance simulating creatine when heated with picric acid, it appears probable that the creatine figures obtained for normal, and for some pathological bloods, represent real creatine. For our creatine determinations we have adopted the procedure of precipitating the blood proteins by dilution of the blood with 4 volumes of 5 per cent trichloroacetic acid. After filtration a portion of the filtrate is treated with hydrochloric acid (5 cc. of 1.0 N acid to 25 cc. of filtrate) and boiled down to a small volume. A little granulated or powdered metallic lead is then added, and the solution taken to dryness and heated on the water bath to expel the hydrochloric acid.<sup>3</sup> The residue is dissolved in water and treated with sodium hydroxide, drop by drop, until a permanent precipitate (of lead hydroxide) is produced. The solution is then made up to a definite volume and filtered. A portion of the filtrate is saturated with dry picric acid and the creatinine determined as usual after filtration. The 10 per cent sodium hydroxide employed here should contain 10 per cent of Rochelle salt.

This procedure gives a satisfactory conversion of pure creatine and is, we believe, reasonably accurate for the creatine determination in normal bloods. While satisfactory for some pathologica

<sup>3</sup> This method is similar to the one recommended by one of us for the determination of creatine in urine (Benedict, S. R., *J. Biol. Chem.*, 1914, xviii, 191.)

bloods, we are inclined to the view that in others no method of creatine determination so far developed can yield results which represent the true creatine content very closely.

When we applied the procedures of heating with alkali and treatment with kaolin as described earlier in this paper, to blood filtrates after heating with acid, and compared the "total creatinine" before and after such procedures, we found that for normal bloods the color-producing substance which resulted from the action of acid was destroyed by alkali and removed by treatment with kaolin exactly as is pure creatinine. Creatine added to blood behaved in a similar manner. These findings hold for beef and for dog blood. We have not yet had sufficient normal human bloods to warrant conclusions concerning them, but we believe that creatine actually exists in such blood.

The findings in connection with the creatine content of blood are of interest also in relation to the question of the nature of the creatinine chromogenic substance in the blood. If this latter substance represents a loosely combined form of creatinine, or some similar compound, we might expect that boiling with acid should convert it into true creatinine. Yet after treatment to convert creatine into creatinine, it is possible by means of the action of alkali or of kaolin, to separate practically quantitatively the creatinine derived from creatine from the chromogenic substance originally present in the blood. Typical results in this connection are presented in Tables IV and V.

The results recorded in Tables IV and V serve to show how differently the creatine of normal blood behaves after conversion to creatinine as compared with the chromogenic substance originally present in the blood. The remarkably sharp separations effected by means of alkali or of kaolin are very striking, and serve to further substantiate our earlier conclusion that preformed creatinine does not exist in blood.

The view that creatine occurs in blood in very appreciable amounts under certain conditions finds confirmation in an isolation experiment, in which creatine was isolated as creatinine-zinc chloride. In this experiment the blood obtained from a dog 46 hours after ligation of both ureters was employed. The total non-protein nitrogen of this blood was 186 mg. per 100 cc., and the blood showed a preformed "creatinine" content of 12 mg., and a

TABLE IV

*Showing the Removal by Kaolin of Blood Creatine and of Creatine Added to Blood After Treatment for Conversion of Creatine to Creatinine, and the Non-Removal of the Original "Creatinine" in the Blood by means of Kaolin after the Boiling with Acid.*

Source of sample.	"Preformed creatinine,"*		"Total creatinine,"		Blood creatine.	Blood creatine removed by kaolin.	Amount of added creatine recovered	Amount of added creatine removed.
	Before kaolin treatment.	After kaolin treatment	Before kaolin treatment.	After kaolin treatment.				
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Beef.	2.67	2.40	4.35	2.22	1.68	1.68+	2.92	2.72
"	2.75		3.75	2.10	0.93	0.93+	3.55	3.75
"	2.22	1.96	3.48	2.04	1.00	1.00		
"	2.22	1.96	3.21	1.70	1.00	1.00		
"	1.21	1.23	3.30	1.59	2.09	1.71		
"	2.67	2.40	4.35	2.22	1.68	1.68		
Dog (ureters ligated).	6.35	2.96	15.30	2.30	8.95	8.95		
Dog (ureters ligated).	4.52	2.02	14.30	1.86	9.78	9.78+		

\* All figures refer to milligrams per 100 cc. of blood, in terms of creatinine.

TABLE V.

*Showing the Destruction by Alkali of the Blood Creatine and of Creatine Added to Blood, after Treatment for Its Conversion to Creatinine, and the Failure of Alkali to Destroy the Original "Creatinine."*

Source of sample.	Blood before creatine conversion.*		Blood creatine.	Blood creatine destroyed.	Added creatine.	Added creatine destroyed.
	Before alkaline heating.	After alkaline heating.				
	mg.	mg.	mg.	mg.	mg.	mg.
Beef.	3.15	3.15	2.60	2.60	3.4	2.00
"	2.18	2.00	2.02	1.40	7.7	5.40
"	2.30	2.30	2.80	2.75	4.58	4.36
"	2.26	2.38	3.09	2.75		
Dog with ligated ureters.	4.08	0.72	5.62	5.41		

\* All figures refer to milligrams per 100 cc. of blood, in terms of creatinine.

creatinine content of 16.8 mg. per 100 cc. The preformed creatinine chromogenic substance was practically completely removed by the use of Lloyd's reagent, while the creatine was unaffected. The filtrate was then boiled with acid and the creatinine content of a portion of the solution determined. The main portion was treated with Lloyd's reagent, which removed the chromogenic substance practically quantitatively. Thus two portions of Lloyd's reagent were obtained, one containing the preformed chromogenic substance of the blood, the second containing the chromogenic substance resulting from boiling with acid. Both portions of the Lloyd's reagent were treated in similar manner to liberate the chromogenic substance, and portions of the solutions thus obtained were analyzed colorimetrically and each was found to contain a chromogenic compound. The solutions were boiled down to very small volumes (about 5 cc.) and washed into small centrifuge tubes with the help of alcohol. The total volume in each tube was about 8 cc.; a few drops of zinc chloride solution were added to each tube, together with a few drops of a mixture of acetic acid and sodium acetate. Within half an hour typical creatinine-zinc chloride crystals began to separate from the tube containing creatinine derived from the creatine in the blood. After standing over night these crystals were centrifuged, washed with alcohol, and dissolved in a few drops of hydrochloric acid, and the solution thus obtained was diluted to a definite volume and analyzed for creatinine colorimetrically. It was found that 10.2 mg. of creatinine had been recovered as the zinc salt, against a theoretical value of 13.6 mg. as determined in a portion of the blood filtrate after hydrolysis. As mentioned earlier in this paper, none of the zinc salt was obtained during 3 days from the tube containing the preformed chromogenic substance in the blood, though addition of a small amount of pure creatinine to the final solution promptly resulted in the separation of typical crystals of the zinc compound. Full details of these isolation experiments will be reported later.

In regard to the question of the occurrence of creatine in blood of patients with renal insufficiency we should state that our results have been so contradictory that we are led to believe that some of these bloods contain large quantities of one or more interfering substances in the creatine determination, while in others the creatine figures may be fairly exact. Some nephritic bloods show

removal of the creatinine derived from creatine by means of kaolin, while others behave very differently. In one case of bichloride poisoning with a very high apparent creatine we failed to effect practically any removal of the chromogenic substance resulting from the action of acid, by means of kaolin treatment. Our results indicate that cases may fall into definite groups in regard to the true creatine content and the study of the question is being continued.

#### DISCUSSION.

Our finding that creatinine does not exist in blood in detectable quantities need not, of course, raise any question as to the value of the determination of the chromogenic substance for clinical or other purposes. In connection with such determination our work has brought out some points which may be of interest. In the first place there is the question of complete saturation of the solutions with picric acid prior to the creatinine determination. This point has been emphasized by Greenwald and McGuire, but we believe that it has not received sufficient recognition. For any method of creatinine determination in blood, except the procedure advocated by Folin and Wu, it is necessary to saturate a solution or mixture with solid picric acid. The Folin-Wu procedure, while perhaps theoretically preferable to the earlier methods from certain standpoints, yields such weak colors that we question the general usefulness of the method. Few analysts can read such colors with even approximate accuracy unless the bloods are very high in chromogenic substance. We therefore believe that saturation with picric acid is preferable in all ordinary work. Such saturation may, of course, be applied to any filtrates (such as the Folin-Wu tungstic acid filtrate) as well as to the original blood.

In any case, we believe it essential that laboratories where attempted saturation with picric acid is a routine procedure should be equipped with shaking machines (a rotary type is satisfactory), and that all solutions to be saturated with picric acid should be placed in such machines for from 5 to 10 minutes, prior to filtration. When numerous bloods are handled it is probable that in no other way can even approximate saturation with picric acid be secured. Where Myers' method, which involves preliminary dilution of the blood with distilled water, is employed, especial care



has to be taken in regard to saturation with the picric acid. In this method the preliminary stirring as recommended by Myers should be followed by from 5 to 10 minutes in a shaking machine. While it is possible to saturate a blood as recommended by Myers, our experience shows that this is rarely accomplished in practice, and that where many bloods are handled simultaneously, it is very difficult to secure saturation by means of stirring. The necessity for full saturation is clearly brought out by Greenwald and McGuire.

A second point of interest in connection with the technique for the chromogenic substance in blood is in connection with the protein precipitant employed and the amount of dilution at the time of the protein precipitation. As noted earlier in this paper, the original method of Folin, employing a 1 to 5 dilution with saturated picric acid when applied to bloods with high "creatinine" values, results in a loss of a very considerable quantity of the chromogenic substance. This loss may amount to 5 mg. or more, per 100 cc. The tungstic acid precipitation, carried out at a 1 to 10 dilution as proposed by Folin and Wu gives a much better recovery of the blood chromogenic substance in the abnormal bloods than does the picric acid method at the dilution of 1 to 5. The figures obtained with this filtrate agree closely with those given by a precipitation with trichloroacetic acid in a 1 to 5 dilution. Figures by these methods are usually appreciably lower than where heat coagulation is employed in a 1 to 5 dilution. The difference, however, is not great, and on account of convenience we should at present recommend use of the Folin-Wu precipitation with tungstic acid, followed by saturation of a portion of the filtrate with dry picric acid in a shaking machine for from 5 to 10 minutes. After filtering from the excess of picric acid the chromogenic substance is determined as in the original Folin method, using standard creatinine solutions in saturated picric acid. Table VI shows some comparative figures for the chromogenic substance in dog bloods rich in this substance where different protein precipitants were employed.

The question naturally arises as to the possible bearing upon theories of creatine and creatinine metabolism of the findings reported in this paper. While we feel that the present data do not warrant detailed discussion, there are certain points brought up by the present work which may be briefly considered.

If creatinine cannot be demonstrated in the blood, there are two possible sources for its presence in the urine. It may be present in the blood in traces, and the kidney may be able to concentrate creatinine from this exceedingly dilute solution. If this view is correct, we should, as pointed out earlier in this paper, be able to demonstrate creatinine in blood after impairment or ablation of the kidney function. No demonstration short of isolation will suffice in this connection. In view of the failure of isolation experiments reported so far, it would seem of interest to discuss the possible origin of the urinary creatinine if creatinine does not occur

TABLE VI.

*Apparent Creatinine by the Different Methods for Blood of Dogs with Ureters Ligated.*

Source of blood.	Hours between operation and withdrawal of blood.	Apparent creatinine in mg. per 100 cc. of blood.			
		Pieric acid method 1:5 dilution.	Sodium tungstate precipitation 1:10 dilution.	Trichloro-acetic acid precipitation 1:10 dilution.	Heat coagulation method 1:5 dilution.
	<i>hrs.</i>				
Dog 1	48	4.85	6.96		
" 2	72	6.55	8.58		
" 3	42	7.35	11.6	11.1	12.0
" 4	46	6.37	8.10	9.04	10.3
" 5	46	11.10	12.24	12.24	14.52

in the blood. Taking this latter view, we must assume that the kidney itself produces creatinine from some precursor substance in the blood. The most probable precursor, from the chemical standpoint, would, of course, be creatine. It is in line with this hypothesis that we find real evidence for the existence of creatine in blood, and it is reasonably certain that under normal conditions the creatine content of the blood is very appreciable. What then is the fate of this creatine? Is it a waste product, or is it to be built up into body tissue? The positive result of our isolation experiment on the creatine of the blood of a dog after ablation of the excretory function of the kidney, where we were able to demonstrate an accumulation of creatine in this blood to an extent exceeding 13 mg. per 100 cc., would seem to show that the creatine of the blood is a waste product, to be eliminated by the kidney.

All five of our dogs in which kidney function was ablated showed high creatine figures as follows: 8.9, 8.1, 9.3, 18.5, and 16.8 mg. per 100 cc. This creatine behaves as true creatine, and was isolated (as creatinine) in the only attempt we have made. Hence we may conclude that creatine accumulates in the blood when the excretory function of the kidney is defective, and we may further conclude that creatine in the blood is a waste product. Creatine is not normally eliminated as such in the urine, hence it would seem that the kidney must eliminate creatine as creatinine or as some other product—perhaps urea. An alternative view that there is some organ which cannot act in destroying creatine except when the kidney is performing its excretory function would seem too extreme a view to deserve consideration. It would seem then that the kidney normally eliminates creatine as creatinine or as some other product. Elimination as creatinine seems far more probable. There are, of course, difficulties in the way of accepting this view. The first which suggests itself is the fact that ingested creatine does not appear in the urine as creatinine except in traces. Most observers except Folin, are agreed that administration of creatine is followed by an appreciable, though very slight increase in the urinary creatinine. The observations of Folin and Denis (7) would explain how it would be possible for ingested creatine to fail to be eliminated in considerable amounts either as creatine or creatinine, even though normally creatine is the source of the urinary creatinine. These investigators have shown that muscular tissue appears to have a marked affinity for creatine, and that this substance rapidly disappears from the blood and is found increased in the muscles. Assuming even an equal affinity for creatine between muscular and kidney tissue, we can readily see why, on account of the relative bulk of the muscular tissue, nearly all of the creatine ingested could get into the muscles as creatine, instead of into the urine as creatinine, even though normally the kidney eliminates the creatine as creatinine. Furthermore, it is quite possible that the formation of creatinine in the kidney represents some definite form of kidney metabolism associated with a portion of its excretory function. Thus creatinine might even represent a measure of a special type of kidney metabolism. It is also possible that the creatine in the circulation differs from ordinary creatine, and is in a combination which can readily be converted into creatinine.

The question of the occurrence of creatine in the urine need not be considered until isolation experiments have shown whether the so called creatine in urine is really that compound. We are studying the question by means of a method which we feel will demonstrate whether creatine occurs in urine.

In the meantime we feel that any theory of creatine and creatinine metabolism must take into account the fact that creatine circulating in the blood appears to be essentially a waste product. The view above proposed attempts to do this, and is the only view so far put forward which takes account of the increased blood creatine after impaired kidney function.

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# A METHOD FOR THE QUANTITATIVE ESTIMATION OF MINUTE AMOUNTS OF GASEOUS OXYGEN AND ITS APPLICATION TO RESPIRATORY AIR.

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## *Historical.*

The principal respiratory gases, oxygen and carbon dioxide, have been measured in animal and plant respiration studies for many years. Except for the absorption of carbon dioxide by alkalis and the method of Winkler for oxygen, physical measurements of volumetric differences following absorption have been used exclusively. Such methods lead to large errors where small quantities of gases are involved. Several different means of measuring carbon dioxide, which depend upon clear-cut chemical processes, have come to be used during the past 10 years. The "biometer" devised by Tashiro (1) made possible a series of investigations by him and his students into the carbon dioxide output of plant and animal tissue under various conditions. Based on these observations they have drawn some rather definite conclusions as to the metabolism of nerve tissue in particular. It is obvious that conclusions with reference to metabolism based on carbon dioxide output would be greatly strengthened and possibly modified if they could be confirmed on the basis of oxygen consumption. In fact, measurements of oxygen consumed under various conditions must serve as far weightier evidence on the mechanism of the respiratory processes than measurements of carbon dioxide or of other possible metabolites alone.

As a sequel to the studies on the carbon dioxide output carried on in this laboratory under the direction of Professor A. P. Mathews, Dr. H. S. Adams, a student of Tashiro, undertook the de-

velopment of a method for measuring the oxygen consumed by respiring tissue. The aim was to determine whether nerve tissue used oxygen gas, and if so, whether the consumption was increased during the passage of the excitatory stages. An apparatus was devised in which oxygen in the presence of nitric oxide was exposed to sodium hydroxide and the nitrite thus formed measured colorimetrically. Preliminary determinations, which were not published, indicated that the principle could be applied in these studies. At this stage Dr. Adams left the work for other duties and the problem was turned over to the writer by Professor Mathews.

The investigations of Child (2), Hyman (3), Lund (4), and Allen (5) on the relationship between respiratory rate and general metabolism have been confined to the lower aquatic organisms where only dissolved oxygen is determined. Winkler's (6) method for this has been used for want of a more direct one. Krogh's (7) gas analysis apparatus adapted so well for gaseous oxygen depends, as do the earlier types of Winterstein (8), Thunberg (9), and others (10), on volumetric measurements before and after absorption of the oxygen. One determination by this method requires more gas than can be employed practically in a single period of respiration for small animals or tissues. Since we were in need of a method for estimating gaseous oxygen in small amounts which would equal the delicacy attained in carbon dioxide estimations, the present method was devised.

Such a method must meet the following requirements: (a) It must permit the withdrawal of successive samples of air from the respiratory chamber and of the analysis of these samples. (b) Successive estimations will give differences representing the oxygen consumed in a given period, and these differences must be of about the same order of magnitude as the carbon dioxide which Tashiro's apparatus detects; *viz.*,  $1 \times 10^{-7}$  gm. (c) The percentage error entering the oxygen estimations must not be larger than that entering the carbon dioxide determinations.

#### *Method.*

The method here presented depends upon the reaction between nitric oxide and oxygen. There is not full agreement as to the nature of this reaction. Raschig (11) explains the reaction as

proceeding in two stages: (a) oxidation of nitric oxide by instantaneously producing  $\text{N}_2\text{O}_3$ ; then, (b) oxidation of the latter by oxygen producing slowly  $\text{N}_2\text{O}_4$ . Wourtsel (12) has good evidence that the reaction is one of the third order,  $\text{N}_2\text{O}_3$  not being an intermediate product, but that  $\text{NO}$  in excess plus  $\text{NO}_2$  yields  $\text{N}_2\text{O}_3$ . In the absence of alkali  $\text{N}_2\text{O}_3$  accumulates to the extent of 2.5 parts per 100 but in presence of alkali does not appear as such. It is agreed that  $\text{N}_2\text{O}_3$  in the presence of sodium hydroxide gives 2 molecules of nitrite and that  $\text{N}_2\text{O}_4$  gives one of nitrite and one of nitrate. Koehler and Marquayrol (13) showed that by having present in the reaction mixture of nitric oxide and oxygen an excess of potassium hydroxide no  $\text{N}_2\text{O}_4$  would be produced. Baudisch and Klinger (14), using solid  $\text{KOH}$  to take up the  $\text{N}_2\text{O}_3$  in this reaction, made volumetric estimations of nitric oxide where excess of oxygen was present, and they suggested the use of an excess of nitric oxide for volumetric estimations of oxygen. In continuing the work of Adams, above referred to, an apparatus and method have been devised in which it is possible to make accurate measurements by means of the following procedure: (a) Running a measured sample of air of unknown oxygen content into an excess of nitric oxide in the presence of sodium hydroxide solution. (b) The colorimetric comparison of the nitrite thus formed with a known quantity of nitrite. (c) Reading off the comparison as grams of oxygen on an empirical curve established by the same procedure, using known quantities of oxygen.

#### *Apparatus and Manipulation.*

The apparatus figured is connected as indicated at four different points with reservoirs (not shown), containing tenth normal sodium hydroxide, nitric oxide, hydrogen, and respiration air, respectively. The arrangement and protection of these are described below. The apparatus is made in separate sections and assembled with heavy rubber connections at points indicated by lines at right angles to the tubing. The mounting is a plane  $\frac{7}{8}$  inch board reinforced with cleats on the back and fashioned to allow uneven glass parts to be set in, and windowed at burettes and reservoirs to permit sighting through. Semirigidity is secured for the assembled parts by fine wire wrapped over rubber around the tubing at convenient points for support and then passed through to the back of the board, where it is twisted to the proper tension.



Numbers refer to stop-cocks and letters to tubes or chambers. *A* is the reaction chamber in which nitric oxide reacts with oxygen in the presence of sodium hydroxide. 2 and tube to *A* are calibrated to hundredths of a cc. for measuring nitric oxide. *B* is a graduated 1 cc. burette with mercury leveling tube of the same bore for measuring samples of diluted air into *A*. *C* is the respira-

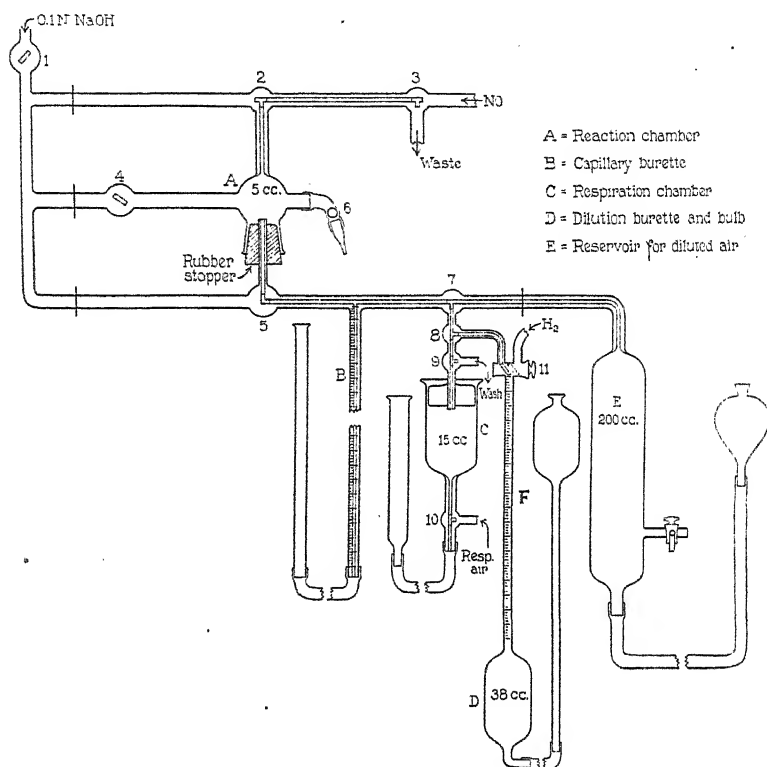


FIG. 1.

tion chamber of adjustable capacity (maximum, 20 cc.) where the tissue under observation is placed on platinum tips which may be used as electrodes for stimulation; the cover is ground and set deeply to provide for sealing with mercury; the side-tube at 10 permits the inflow of air to be used as respiration medium; the side-tube at 9 permits the outflow of wash air. This chamber during standardization of the apparatus serves as a reservoir for

oxygen. *F* is a graduated 3 cc. burette sealed below to a calibrated bulb of 38 cc. capacity and with a leveling tube of the same bore. This burette and its leveling tube are parts from the Van Slyke amino nitrogen micro apparatus. It serves for diluting respiration air drawn in through 9, 8, 11, and provides storage space or permits its transfer to *E* through 8, 7 for storage. Ground points must be kept clean and tight. Cleaning may be effected by removing grease from joints then applying suction at 3, 6, and 9 successively, using first 30 per cent nitric acid when mercury particles are lodged with grease, then following with water, alcohol, and ether. Tightness of joints is secured by using sparingly a heavy grease of 2 parts gum rubber to 1 of vaseline with such an amount of paraffin oil (4 to 15 drops to 25 cc.) as is required to make the mixture smooth. In warm weather less oil is necessary than in cold.

To make an estimation the sample of air to be analyzed is secured in *C*. If it is from an outside source it may be drawn in through 9 by adjusting the leveling tube, *C* having been filled previously with mercury up to 9 and its outlet. If it is respiration air, the air may enter at 10, bubbling through such mercury in *C* as may have been used to adjust its capacity. In order to bring the oxygen concentration to the neighborhood of 0.3 to 0.9 per cent by volume, if dilution is necessary, a portion of it may be measured into *F* through 9, 8, 11 and then diluted to the mark on *D* with hydrogen through 11. This is then run into *E* under pressure where after complete mixing it may be held for analysis. Before analysis the system 1, 2, 3, 4, 5, *A*, 6 must be filled completely with tenth normal sodium hydroxide; the system *B*, 7 to 5 on the one hand and *E* on the other must be filled with mercury. The necessary quantity of nitric oxide is measured through 2 and driven into *A* by alkali through 2, 6 being opened each time an inlet into *A* is opened. From *E* a sample of air is taken into *B* and, with 7 closed, measured out into *A*, that in the tube above 5 being then driven into *A* by alkali through 5. The reaction progresses to equilibrium and the products are driven out through 6 by alkali entering 2, 4, and 5, care being taken to keep the residual gas in *A*. From 6 the products are run directly into a volumetric flask containing the reagents used for developing the dye. The intensity of color developed as measured against a standard in a colorim-

eter becomes a measure of the quantity of oxygen entering into the reaction.

### *Materials.*

Oxygen for this work is generated by the treatment of hydrogen peroxide with manganese dioxide in alkaline solution. A nitrometer fitted with a capillary stop-cock at the top, a leveling tube below, and a side-tube with a stop-cock and funnel attached, is filled with 5 per cent sodium hydroxide. A few cc. of hydrogen peroxide are run into the nitrometer through the side-tube and a minute amount of manganese dioxide is then washed in with the alkali. The oxygen is drawn off through the capillary stop-cock into the apparatus for dilution.

Tenth normal sodium hydroxide is sufficiently concentrated to take up quickly the products of the oxygen—nitric oxide reaction. This is prepared oxygen-free by boiling redistilled water, adding slowly the necessary amount of pure, solid sodium hydroxide to it at the boiling temperature, and cooling in a stream of oxygen-free hydrogen. The cooled alkali is then drawn by suction into its reservoir on the apparatus. The reservoir for several days previously is treated with frequent changes of oxygen-free hydrogen to remove as completely as possible the oxygen occluded on its walls. In spite of these precautions against the admission of oxygen into the apparatus with the alkali, unless further and continued treatment of it is employed, traces continue to appear. These last traces may be made so small as to be undetectable, by adding through a dropping funnel into the reservoir a few drops of 10 per cent manganous chloride solution. The white manganous hydroxide flocks down and appears as the black dioxide as rapidly as the traces of oxygen in the alkali react with the former. The presence of unchanged manganous hydroxide after a few hours is evidence that oxygen is absent, and a blank run on the reagent will yield no color. The delivery of this solution to the apparatus is by siphon and pressure from an 8 liter bottle that is kept two-thirds filled. The bottle is supplied by a carboy placed on the floor. Pressure, to insure delivery from carboy to bottle, is secured from a commercial tank of electrolytic hydrogen (99.9 per cent) which is bubbled through a train of two alkaline pyrogallol solutions and one of boiled linseed oil. This replaces the alkali withdrawn by an oxygen-free atmosphere.

The diluent for the oxygen and for respiration air is hydrogen. Nitrogen has been used with equal success in every particular, but that secured commercially requires greater pains and more time for ridding it of substances that cause color to develop in otherwise blank mixtures. The relatively pure commercial hydrogen saves time and furnishes the desired pressure. This is washed, as when used for pressure on the tenth normal sodium hydroxide, through two alkaline pyrogallol solutions and one of boiled linseed oil to remove oxygen, traces of carbon monoxide, and ozone, and again through an alkaline pyrogallol solution. Thus prepared it shows no trace of oxygen when tested by the method under consideration.

Nitric oxide is prepared in a Kipp generator by the reduction of nitric acid by copper. Nitric acid of 35 per cent concentration and copper turnings are used. The gas is washed twice through 20 per cent sodium hydroxide to take out other active oxides of nitrogen and is stored in the apparatus over alkali of the same concentration in such a way that alkali replaces the gas as it is withdrawn. Thus prepared it is between 81 and 85 per cent pure as determined by its absorption in alkaline permanganate in the Van Slyke apparatus for amino nitrogen. The diluents present are inert as far as our reaction is concerned. This concentration proves, for the conditions under which analyses here reported have been made, to be the most suitable.

A colorimetric method demands a standard color which can be duplicated with accuracy under the rather variable conditions of temperature and pressure which prevail at the time of the experiments. The nitrite reagents described by Woodman and Norton (15) are used. The sulfanilic acid and  $\alpha$ -naphthylamine in acetic acid solution are prepared in liter quantities and stored separately in amber bottles with siphon attachment. 10 cc. of equal parts of the mixed reagents are run into a 50 cc. volumetric flask, then 5 cc. of the diluted sodium nitrite, to be described later, and finally tenth normal sodium hydroxide to bring the volume to 50 cc. This is mixed, allowed to stand 30 minutes, and compared in the Duboseq colorimeter with the ultimate standard (permanganate-dichromate mixture) set at 10 mm., or with the unknown when the former nitrite standard is set at 20 mm. on the scale. The analyzed grade of sodium nitrite put up by the Baker Chemical Co., proved satisfactory. Stock solutions were prepared from this on

three different occasions during June and July of 1918 and dilutions were made at the same time to contain 8.892 mg. of sodium nitrite per liter. Both stocks and dilutions 1 year later were of the same concentration of nitrite as measured against fresh preparations and against the ultimate standard consisting of a mixture of 60 parts M/1,000 potassium permanganate and 40 parts M/1,000 potassium dichromate (16), measurements being made in a Duboseq colorimeter. The difficulty of using the last as a standard in routine work is that a tinge of purple not present in the diazo compound gives a different quality to the color and thus renders comparisons slow and difficult. Furthermore, the change in the color intensity of the permanganate-dichromate mixture with variations of temperature, differs from that of the diazo dye.

The empirical relation between intensity of color and quantity of oxygen, is expressed on the curve (Fig. 2) in which colorimeter readings are found on the ordinates and grams of oxygen on the abscissæ. Colorimeter readings are thus inversely proportional to intensity of color. And the depth of color is a measure of the quantity of nitrite present. Since the nitrite formed, as will be shown later, is a function within rather narrow limits of the concentration of nitric oxide, it is not a measure of the quantity of oxygen except where the concentration is kept very nearly constant. But, this being done, the intensity of color produced may be taken as a measure of the quantity of oxygen in the sample. The colorimeter reading from a sample of unknown oxygen content is located on the curve, and the grams of oxygen corresponding thereto is read off directly.

It has been found in the course of the estimations that the intensity of color, when the same amounts of oxygen are used successively, is a function of the concentration of nitric oxide. It is therefore necessary to keep this constant within certain limits in all estimations. This is done by measuring off the quantity of 85 per cent nitric oxide to be used, knowing beforehand very roughly the quantity of air and its oxygen content. One preliminary trial will settle this for any single gas. Thus in the residual gas, after the completion of the reaction, the nitric oxide concentration should be approximately constant for all estimations, and this concentration should be just so low that nitric oxide will be dis-

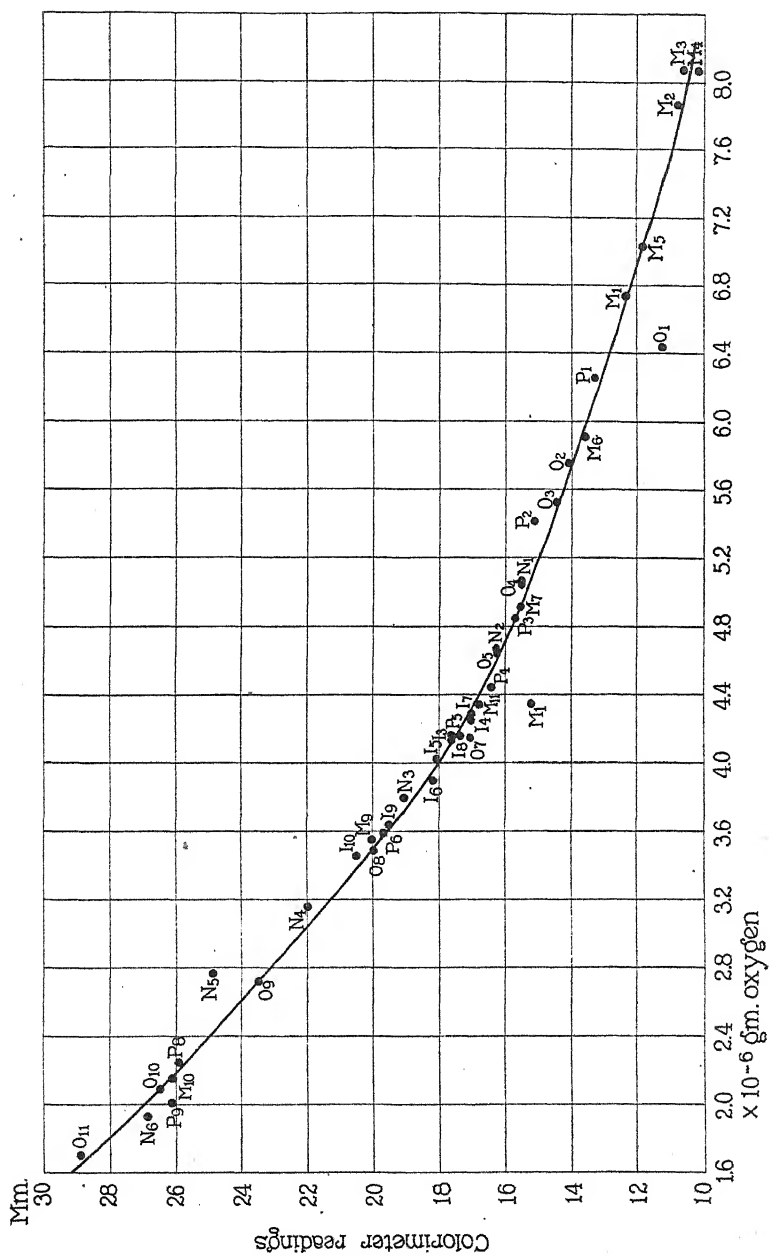


FIG. 2.

TABLE I.  
Data Used in Establishing Curve.

Temperature.	Barometer.	NO used.	"Air" used.			O <sub>2</sub> calculated in sample.*	Standard at 20; average of three colorimeter readings.*
°C.	mm.	cc.			cc.	gm.	mm.
20.5	740.5		I	3	0.895	$4.148 \times 10^{-n}$	17.6
20.5	740.5			4	0.913	4.232	17.0
20.5	740.5			5	0.867	4.018	18.0
22.0	731.5	0.110±		6	0.852	3.881	18.2
22.0	731.5	0.010		7	0.938	4.273	17.1
22.0	731.5			8	0.915	4.168	17.4
22.0	731.5			9	0.797	3.630	19.5
22.0	732.0			10	0.758	3.455	20.5
23.0	736.0	0.103	M	1	0.651	6.738	12.4
23.0	736.0	0.103		2	0.759	7.856	10.8
23.0	736.0	0.103		3	0.785	8.073	10.7
23.0	736.0	0.135		4	0.780	8.073	10.2
23.0	736.0	0.135		5	0.678	7.018	11.9
23.0	736.0	0.116		6	0.572	5.921	13.7
23.0	736.0	0.116		7	0.475	4.916	15.6
23.0	736.0	0.116		8	0.422	4.368	15.1
23.0	736.0	0.103		9	0.345	3.571	20.0
23.0	736.0	0.103		10	0.209	2.163	26.1
22.0	746.5	0.103	N	11	0.412	4.340	16.7
23.0	745.0	0.103		1	0.725	5.063	15.5
23.0	745.0	0.103		2	0.666	4.651	16.3
23.0	745.0	0.103		3	0.543	3.792	19.1
23.0	745.0	0.103		4	0.453	3.163	22.0
23.0	745.0	0.087		5	0.397	2.772	24.9
22.0	745.0	0.087		6	0.274	1.920	26.8
23.0	747.0	0.113	O	1	0.830	6.419	11.5
23.0	747.0	0.113		2	0.747	5.777	14.2
23.0	747.0	0.113		3	0.713	5.514	14.4
22.0	747.0	0.103		4	0.651	5.052	15.5
22.0	747.0	0.103		5	0.601	4.664	16.3
22.0	747.0	0.103		6	0.553	4.291	Leak developed.
23.0	747.0	0.103		7	0.534	4.130	17.0
24.0	747.0	0.103		8	0.453	3.492	19.7
23.0	747.0	0.087		9	0.350	2.707	23.5
24.0	747.0	0.087		10	0.270	2.081	26.5
23.0	747.0	0.087		11	0.221	1.709	28.9
23.0	747.0	0.087		12	0.140	1.083	31.2

TABLE I—*Concluded.*

Temperature.	Barometer.	NO used.	"Air" used.			O <sub>2</sub> calculated in sample.*	Standard at 20; average of three colorimeter readings.*
°C.	mm.	cc.	P		cc.	gm.	mm.
22.0	745.0	0.113	P	1	0.881	$6.255 \times 10^{-6}$	13.4
22.0	745.0	0.113		2	0.764	5.424	15.2
22.0	745.0	0.113		3	0.681	4.835	15.8
22.0	745.0	0.113		4	0.625	4.437	16.4
21.0	745.0	0.103		5	0.584	4.160	17.6
21.0	745.0	0.103		6	0.504	3.590	19.6
21.0	745.0	0.087		7	0.489	3.483	20.0
21.0	745.0	0.087		8	0.315	2.244	25.9
22.0	745.0	0.087		9	0.283	2.000	26.1
23.0	745.0	0.087		10	0.140	0.991	37.9

\* The last two columns furnish the data for the construction of the curve.

*Preparation of "Airs" Referred to in Table I.*

"Air."	Date.	At time of dilution.		O <sub>2</sub> diluted.	Diluted with H <sub>2</sub> to	O <sub>2</sub> in air as used.
		Temperature.	Pressure.			
	1918	°C.	mm.	cc.	cc.	per cent
I	Nov. 5			0.782	218.5	0.3579
M	Dec. 24	21.0	735	0.620	76.45	0.8110
N	" 27	22.5	745	0.415	76.78	0.5405
O	" 28	23.0	747	0.463	77.55	0.5970
P	" 28	23.0	747	0.420	76.69	0.5477

solved by the tenth normal sodium hydroxide to so small an extent that on withdrawal into atmospheric air, an undetectable quantity of nitrite will be formed by reaction with atmospheric oxygen as shown by absence of color when the reagents are added. Such blank tests must be made from time to time to assure one that too large quantities of nitric oxide are not being used. And to give assurance that enough nitric oxide is being used, it is only necessary to add a small quantity of the air under observation to the residual gas and to withdraw the alkali through it into the reagent when slight coloration will result.

The effect of temperature upon the intensity of color has been found to be negligible within 5°C. of the temperature prevailing in the laboratory (23°C.) at the time the curve was constructed.



TABLE II.

*Data on Airs of Unknown Oxygen Content.*

Unknown.	Diluted to	O <sub>2</sub> found in sample.	Total O <sub>2</sub> found.	Total O <sub>2</sub> present.	Error.
	cc.	gm.	cc	cc.	per cent
I	77.888	6.43 × 10 <sup>-6</sup> 5.99 5.49 5.24 4.27 3.90	0.440 0.417 0.428 0.431 0.458 0.459		
Average reported.....			0.439	0.450	-2.4
II	76.018	7.14 × 10 <sup>-6</sup> 5.43 4.47 4.12	0.467 0.501 0.504 0.506		
Average reported.....			0.494	0.475	+4.0
III	77.137	6.17 × 10 <sup>-6</sup> 4.07 4.62 3.32 3.29 4.32 4.18	0.377 0.398 0.364 0.387 0.403 0.367 0.398		
Average reported.....			0.385	0.371	+3.8
IV	77.315	6.83 × 10 <sup>-6</sup> 4.55 3.93 3.36 2.93 2.36	0.414 0.412 0.409 0.409 0.413 0.405		
Average reported.....			0.410	0.406	+0.98

*Tests of Method.*

After the establishment of the curve, using measured quantities of oxygen mixed with known quantities of diluent, the next step consisted in ascertaining how reliable the method would be in estimating unknown quantities of oxygen. A colleague measured

off in the instrument at different times four samples of pure oxygen prepared as described above, mixed them with hydrogen separately and left them with the writer for analysis. The table presenting the results of the analyses shows a maximum variation of 4.0 per cent between the quantities given and the quantities reported. This is a greater variation than is to be expected using a modified technique now employed and described herewith for insuring proper mixing of gases in the storage chamber. Samples of considerable variation in size were used to see that the individual values secured, within the range of the curve, were well within the limits of error found in the reports. Under "Unknown IV" (Table II) the values show typical variations to be found among separate determinations of samples analyzed subsequent to the date of this report.

#### *Application of Method.*

After confirming the relative accuracy of the method on artificial air mixtures, it was next applied to airs before and after respiration. The procedures for the analysis of samples of respiration air were identical with those described above. The tissue used was the excised sciatic nerve of the frog, *Rana pipiens*. The brain was pithed and one or both sciatics removed according to the experiment. Removal was as rapid as possible and with the least possible trauma to the nerve. The excised nerve was placed at once in Ringer's solution, removed gently to dry on washed filter paper, weighed, and placed in a respiration chamber. Prepared respiration air was passed through the chamber for such a time as experience has shown sufficient to have washed it completely of air previously contained. A nerve was considered as "resting" or stimulated according to treatment—"resting" if prepared with sharp instruments and handled speedily and gently and placed on uncharged electrodes of the chamber; stimulated if prepared in the same way, but treated through electrodes with rapid induced shocks which were shown in the muscle-nerve preparation of the other leg, to be just sufficient to cause response of the muscle when applied to the proximal end of the nerve, stimulation being continued through the period of respiration. Samples of air taken from the empty chamber before and after use of the same strength of induced shocks for the same period, showed the

TABLE III.

*Data on the Respiration of the "Resting" Sciatic Nerve.*

Date.	Temperature.	Barometer.	Brain pithead.	Sciatic nerve removed.	Weight of nerve.		Time of respiration.		Volume of respiration air.	Dilution of respiration air.	
					Before.	After.	Beginning.	Close.		Before.	After.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
1919	°C.	mm.	p.m.	p.m.	mg.	mg.	p.m.	p.m.	cc.	cc.	cc.
Jan. 11	22.5	756	5.05	5.13	63	60	5.18½	5.38½	19	2.198 to 38.82	2.470 to 37.66

*Analysis of "Airs" from the Last Two Columns of Table III.*

Air.	Temperature.	Barometer.	NO	Air used.	Average of three col- orimeter readings.	O <sub>2</sub> as read off on curve.	O <sub>2</sub> in 19 cc. respiration air.
	°C.	mm.	cc.	cc.	mm.	gm.	gm.
Unrespired, diluted as per Column 11 above.	24.5	742	0.103	0.900	19.2	3.75 × 10 <sup>-6</sup>	1.363 × 10 <sup>-3</sup>
	24.0	742	0.103	0.708	22.7	2.97	1.374
	24.0	742	0.103	0.745	22.1	3.10	1.363
	24.0	742	0.103	0.869	20.4	3.46	1.305
	24.0	742	0.103	0.997	18.3	3.97	1.305
Average.....							1.342 × 10 <sup>-3</sup>
Respired, diluted as per Column 12 above.	23.0	742	0.103	0.966	17.1	4.29 × 10 <sup>-6</sup>	1.260 × 10 <sup>-3</sup>
	23.0	742	0.103	0.801	20.5	3.44	1.219
	23.0	742	0.103	0.870	Withdrawn too early.		Discarded.
	23.0	742	0.103	0.951	17.3	4.23 × 10 <sup>-6</sup>	1.262 × 10 <sup>-3</sup>
	23.0	742	0.103	0.684	22.5	3.02	1.253
	23.0	742	0.103	0.732	21.4	3.25	1.260
Average.....							1.251 × 10 <sup>-3</sup>

same oxygen content; thus showing that the current alone was not responsible for changes in oxygen content. Mechanical stimulation resulting from trauma by rough handling or drying of nerves, gave oxygen consumptions higher than those for the "resting" nerves. The effect of mercury vapor on the respiration of the

nerve was not eliminated entirely, though since the "resting" and stimulated nerves were treated alike in exposure to the mercury, the relative values between "rest" and stimulation remain comparable.

The choice of the partial pressure of oxygen to be used in the respiration medium, will depend upon the nature of the tissue which is subjected to experimentation. For analysis it may be diluted as required. In the experiments here cited atmospheric air with carbon dioxide removed, was diluted with hydrogen to give an oxygen concentration of about 5 per cent by volume, or a partial pressure of approximately 35 mm. of mercury which is that given by Krogh (17) for human arterial blood.

To secure the figures in the last column of Table III the following formula was used:

$$\frac{\text{Gm. O}_2 \text{ read on curve} \times \text{vol. diluted air} \times \text{vol. resp. air} \times \text{wt. O}_2 \text{ per cc. time of analysis}}{\text{Wt. O}_2 \text{ per cc. time of dilution} \times \text{vol. air used in analysis} \times \text{vol. air taken for dilution}}$$

Taking values before respiration and again after, we have,  $1.342 \times 10^{-3} - 1.251 \times 10^{-3} = 0.091 \times 10^{-3}$  gm. of  $\text{O}_2$  used by 60 mg. of nerve tissue in 20 min. "resting," or  $0.76 \times 10^{-5}$  gm. of  $\text{O}_2$  used by 10 mg. in 10 min. Another nerve prepared in the same way, "resting," used  $0.434 \times 10^{-5}$  gm. of  $\text{O}_2$  per 10 mg. per 10 min.

Three similar nerves from different frogs under stimulation with a weak induced current used 1.32, 1.39, and  $1.51 \times 10^{-5}$  gm.<sup>2</sup> of  $\text{O}_2$  per 10 mg. per 10 min., respectively.

The writer was not equipped at the time to make oxygen and carbon dioxide estimations simultaneously for the purpose of checking gaseous intake against gaseous output.

#### CONCLUSIONS.

1. A microchemical method for the estimation of oxygen has been devised depending upon the colorimetric estimation of nitrite formed in the interaction of oxygen and nitric oxide in the presence of sodium hydroxide solution.

2. This method is sufficiently delicate for measuring amounts of oxygen of the order of magnitude  $1 \times 10^{-7}$  gm., or less than 0.1 c.mm. of the gas.

3. Application of the method has been made to the micro respiration of frogs sciatic nerves and it has been found that from 0.434 to  $0.76 \times 10^{-5}$  gm. of oxygen is used by 10 mg. of nerve per 10 min. in the "resting" state and from 1.32 to  $1.51 \times 10^{-5}$  gm. when stimulated by weak induced shocks.

The writer wishes here to express his appreciation of the interest and assistance given by Professor F. C. Koch throughout the course of this investigation.

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# THE DIFFERENTIAL PRECIPITATION OF THE PROTEINS OF COLOSTRUM AND A METHOD FOR THE DETERMINATION OF THE PROTEINS IN COLOSTRUM.

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As a result of work on the appearance and disappearance of protein in the blood of young calves (1) it became desirable to determine the proteins in colostrum. The blood of a new-born calf after it has taken colostrum contains rather large quantities of euglobulin and pseudoglobulin I whereas before it has taken colostrum these proteins are absent. The evidence points to a direct absorption of the protein from the alimentary tract for two reasons, (a) the time interval between ingestion of colostrum and the appearance of the proteins in the blood is short, 2 to 5 hours, and (b) these proteins do not appear to any extent in the blood of calves which are fed milk from a cow well along in lactation. If the assumption of direct absorption is correct, and it appears to be, we should expect to find both types of globulin in colostrum.

Two groups of investigators, Crowther and Raistrick (2), Dudley and Woodman (3), and Woodman (4), working upon the relation of the proteins of milk and colostrum to each other and to similar proteins of other species or to blood have attempted the separation of euglobulin from the other globulins of colostrum. Crowther and Raistrick precipitated casein<sup>1</sup> with potassium alum according to the procedure of Schlossmann (5), filtered, neutralized the filtrate, precipitated the globulins with magnesium sulfate, and then dialyzed the dissolved precipitate to separate the euglobulin from the pseudoglobulin. Dudley and Woodman precipitated the

<sup>1</sup> The term casein is used in this work to designate the protein of milk which corresponds to the English term caseinogen.

casein of colostrum with acetic acid, neutralized the filtrate, and then separated the globulins in the same manner as did Crowther and Raistrick.

Various procedures have been suggested for the determination of casein, albumin, and total globulins in colostrum. The literature on the subject has been reviewed by Engel (6). Sebelien (7) obtained maximal values for globulin in colostrum by subtracting the value obtained for casein by precipitation with acetic acid from that obtained by precipitating with magnesium sulfate; magnesium sulfate precipitates casein and all of the globulins. A minimal value was obtained by saturation with sodium chloride which precipitates casein and part of the globulins. From our work the minimal values obtained by Sebelien probably represent the euglobulin and not any of the pseudoglobulins. Albumin was determined in the filtrate from the saturated magnesium sulfate precipitation. Tiemann (8) utilized the clay plate method of Lehmann (9) to separate the dissolved proteins of colostrum from the undissolved proteins. Results were obtained for casein which agreed very well with those obtained by the methods of Schlossmann and of Sebelien. Simon (10) has reexamined the procedures of previous investigators for the analysis of milk and obtained similar results for casein with acetic acid and alum and higher results with saturated magnesium sulfate and sodium chloride, due, as he suggests, to the precipitation of globulin by these salts. For total protein Almén's tannic acid reagent, phosphotungstic acid, and trichloroacetic acid gave essentially the same results.

*The Basis for the Determination of the Proteins of Colostrum.*—The general technique for the determination of the proteins of colostrum involves the direct determination of casein and the estimation of the remaining proteins by means of definite concentrations of a salt or by the use of different salts. In this work anhydrous sodium sulfate was used. Storch (11) has used sodium sulfate at room temperature in the separation of the proteins of milk. Casein was precipitated by saturated sodium sulfate, which at room temperature would be equivalent to between 14 and 19 per cent (16–20°C.). This corresponds very closely to the precipitation limits of casein found by us. Three procedures were open: Procedure A, to precipitate the casein with an acid or alum and and then to add various amounts of sodium sulfate to the neutra-

lized filtrate, which is essentially the method of Crowther and Raistrick and Dudley and Woodman in preparing the proteins of colostrum and milk, Procedure B to precipitate casein and euglobulin together with saturated sodium chloride and from a series of precipitations with sodium sulfate on other portions of colostrum to calculate the amount of casein and euglobulin present, or Procedure C to make a series of precipitations with sodium sulfate and to precipitate the casein from the filtrates of such precipitations. It was hoped that all three procedures could be utilized in establishing one of them as the most convenient and reliable but this hope was not entirely realized. On the other hand each procedure helped in the analysis of the others. Experiments with colostrum in which Procedure A was used were variously successful and unsuccessful. The chief difficulties were the determination of the completion of precipitation of casein and the altered conditions as the result of acidification and neutralization. Procedure B was also more or less unsuccessful but the results served as a fair check on the determinations of casein and euglobulin. Procedure C has yielded reasonably consistent results.

#### EXPERIMENTAL.

The experimental work on the determination of the proteins of colostrum involved two phases, (a) the fractionation of colostrum with sodium sulfate and (b) the determination of casein. As the result of previous experience with blood (12) the analytical procedures were confined almost entirely to the analysis of small quantities of colostrum, 0.5 cc. diluted with 15 cc. of solution or an equivalent dilution. The measurement of such small quantities of the most concentrated samples of colostrum presented certain difficulties on account of the viscosity of the material; we were able, however, to obtain results which agreed very closely with those found with larger quantities. All samples of colostrum were centrifuged to remove most of the fat, the colostric bodies, etc.; our remarks, therefore, apply only to the "fat-free" colostrum. When sodium sulfate was used it was added to the measured sample of colostrum in concentrations which would give the desired percentage in the volume of colostrum used plus the volume of added solution. Most of the precipitations were made by the



addition of known concentrations of sodium sulfate to measured quantities of colostrum, such results were often checked by adding the salt to the diluted colostrum. The degree of dilution adopted was that found to be sufficient for blood. That the dilution was adequate was verified by experiments in which 0.5 cc. of colostrum of high protein content was added to 30 cc. of salt solution; *i.e.*, double the dilution employed in this work. All precipitations were made at 37°C. For the precipitation of casein a 10 per cent solution of acetic acid was used or a saturated solution of potassium alum. The quantity of protein precipitated was estimated by analyzing an aliquot portion of the filtrate by the Kjeldahl method. Because of the amount of material to be oxidized the usual 500 cc., or 250 cc., Kjeldahl flask was used in place of the Pyrex test-tubes employed in the work on blood, 10 cc. of sulfuric acid were used in the oxidation. With suitable precautions it is possible, however, to use the large test tubes and the micro distillation procedure introduced by Folin.

*Effect of Increasing Concentrations of Sodium Sulfate upon Colostrum or Milk.*—Colostrum was treated with various concentrations of sodium sulfate to determine the presence of critical zones in the curve of protein precipitated. In Tables I and II are data from experiments on colostrum and also on milk.

Sodium sulfate was added to diluted colostrum, or solutions of a given concentration to measured quantities of colostrum, so that a series of solutions which differed from each other by a small but definite amount of the salt was obtained. When precipitation occurred the filtrates were analyzed for nitrogen by the Kjeldahl method and the quantity of protein was estimated from the total nitrogen content of the colostrum. Examples of such series are contained in Tables I and II. In Table II analyses of a number of different samples of colostrum are given. These data are presented to show the nature of the deviations from the conclusions which may be drawn and also to bring out the constancy with which other phenomena occur.

Colostrum, like blood, is a mixture of proteins and other substances. Any procedure which attempts to separate the proteins of such a mixture without purification must, indeed, carry with it certain errors such as may result from adsorption, possible

overlapping of precipitation limits, etc. To obtain a relative degree of constancy in any set of procedures is, we believe, to approach the minimum of error under the conditions imposed. At present our methods for the separation of proteins are confined to precipitation with salts or acids, and occasionally alcohol.

TABLE I.

*Nitrogen Precipitated from Colostrum and Milk by Acidification, Saturated Sodium Chloride, and Various Concentrations of Sodium Sulfate.\**

	Colostrum.				Milk.	
	N in filtrate.	N precipitated.	N in filtrate after acidification.	N precipitated by acid.	N in filtrate.	N precipitated.
Total N.....	3.226				0.551	
Alum.....	2.363	0.863			0.144	0.407
Acetic acid.....	2.408	0.818			0.124	0.427
Saturated NaCl.....	0.808	2.418				
Na <sub>2</sub> SO <sub>4</sub>						
13.5 per cent.....	1.992	1.234	1.159	0.883	0.553	0.007
14.0 " ".....	1.926	1.300	1.056	0.870	0.540	0.011
14.2 " ".....	1.860	1.366	1.002	0.858		
14.5 " ".....	1.383	1.843	0.726	0.657		
16.4 " ".....	0.682	2.542	0.429	0.283	0.322	0.229
17.4 " ".....	0.508	2.718	0.379	0.129	0.153	0.398
18.4 " ".....	0.445	2.781	0.338	0.107	0.136	0.415
19.3 " ".....	0.437	2.789	0.338	0.099	0.132	0.419
20.3 " ".....	0.429	2.977	0.330	0.099		
21.3 " ".....	0.392	2.834	0.214	0.178	0.128	0.423
23.2 " ".....	0.396	2.830	0.190	0.206	0.128	0.423

\* Results are expressed as grams of nitrogen in 100 cc. of the original sample.

*Protein, Euglobulin, Precipitated at 14.0 to 14.2 Per Cent of Sodium Sulfate.*—From a consideration of the data presented in the tables it will be seen that there is, in general, a marked change in the quantity of protein precipitated between 14.2 and 14.5 per cent of sodium sulfate and that the quantity of protein precipitated at 14.0 to 14.2 per cent of sodium sulfate is approximately the same. The point in the curve of precipitation at which there is little change in the quantity of protein precipitated will be desig-

nated the "critical zone." A sharp critical zone is not evident when the concentrations of sodium sulfate differ by 1 per cent of sodium sulfate. If the results obtained with a difference of 1 per cent be plotted the points representing protein precipitated approximate a straight line between 12.5 and 16 per cent of sodium sulfate with a very sharp break between 16 and 17 per cent. If, on the other hand, the results from a series of precipitations in which the sodium sulfate differs by 0.2 per cent be plotted there is almost invariably a zone between 13.5 and 14.5 per cent of sodium sulfate in which an increment of 0.2 per cent does not cause a marked increase in the quantity of protein precipitated. The critical zone usually occurs at 14.0 to 14.2 per cent of sodium sulfate. There are one or two exceptions in Table II. In the case of Sample F the critical zone occurs at 13.5 to 14.0 per cent of sodium sulfate and in the case of Sample E the critical zone is at 14.2 to 14.5 per cent.

Data obtained after the removal of casein with acid and then the addition of sodium sulfate to the neutralized filtrate did not always agree with that obtained by direct precipitation with regard to the absolute amount of protein precipitated. Whether the absolute results agreed or not the evidence of a critical zone remained and with less variation than with direct precipitation. The failure always to obtain agreement in the quantity of protein precipitated is to be laid, we believe, to the effects of acidification and neutralization. No matter what procedure is adopted the results obtained at 18.4 per cent of sodium sulfate essentially agree. In one attempt to solve the difficulties in this procedure a series of experiments was conducted in which various amounts of acetic acid were added to attain certain degrees of acidity or flocculation. After filtration the neutralized filtrates were precipitated with sodium sulfate. It was found that, in spite of the fact that various quantities of protein were precipitated by acetic acid and subsequently by 14.0 per cent of sodium sulfate, the sums of the quantities of protein precipitated by acetic acid and 14.0 per cent of sodium sulfate were practically identical and equalled the protein precipitated by sodium chloride. Furthermore, the quantity of protein precipitated by 18 per cent of sodium sulfate was essentially the same. The difficulties of this procedure with the possibilities for

error do not seem to warrant its use for the determination of the proteins of colostrum.

A critical zone at 14.0 to 14.2 per cent of sodium sulfate agrees with the results obtained with blood.<sup>2</sup> In our discussion protein precipitated up to and including the critical zone will be designated euglobulin. From the work on the determination of casein, presented below, it seems that very little, if any, casein is precipitated until 14.5 per cent of sodium sulfate has been added. Contamination of the protein precipitated at the critical zone would appear to consist essentially of pseudoglobulin I. Preparations of the protein precipitated at 14.0 to 14.2 per cent retain their precipitation reactions at these percentages, they are precipitated by dialysis and are soluble in salt solution. It may be added that under the conditions of dialysis employed a rather large percentage of salt-insoluble protein was obtained which was difficultly soluble in dilute acid or alkali in the cold.

In the case of colostrum it was not possible to verify the assumption that the protein precipitated at 14.0 to 14.2 per cent of sodium sulfate was euglobulin, as was done in the case of blood by the use of other precipitants such as carbon dioxide and saturated sodium chloride, since both euglobulin and casein are, in part at least, precipitated by carbon dioxide or saturated sodium chloride. Data obtained by the use of saturated sodium chloride agree approximately with the sum of the euglobulin nitrogen and the casein nitrogen. In Table II are given results for euglobulin obtained by subtracting the casein nitrogen from the nitrogen precipitated by saturated sodium chloride. Carbon dioxide as a precipitant of euglobulin plus casein gave rather variable results. From diluted milk casein is completely precipitated. With colostrum carbon dioxide apparently precipitated casein and sometimes euglobulin, at least the analytical results agree with those obtained either by acetic acid precipitation or by sodium chloride precipitation. It was thought that possibly by removal of the calcium with

<sup>2</sup> In the case of blood the limits were given as 13.5 to 14.5 per cent of sodium sulfate. From the work here presented and subsequent work on blood we would suggest 14.2 per cent of sodium sulfate as best single concentration to use; such a concentration represents an approximately molar solution of sodium sulfate.

an oxalate or citrate consistent results could be obtained, but without success.<sup>3</sup>

*The Presence of Critical Zones for Colostrum at 17.4 to 18.4 and at 21 to 22 Per Cent of Sodium Sulfate.*—A second critical zone is evident for both colostrum and milk at approximately 17.4 to 18.4 per cent of sodium sulfate. At these concentrations, reasoning by analogy with blood and from data obtained with milk and purified solutions of casein, pseudoglobulin I and casein are completely precipitated. Preparations of casein are precipitated completely at 18.0 per cent of sodium sulfate and dialyzed preparations of pseudoglobulin I are precipitated at 17.4 per cent of sodium sulfate.

Data obtained by fractioning the neutralized filtrate from colostrum after the precipitation of casein with acetic acid give a critical zone at 16.4 to 17.4 per cent of sodium sulfate, corresponding with the zone obtained in blood. Casein, however, appears to require a slightly higher concentration of sodium sulfate, 18.0 per cent, to insure complete precipitation under all conditions. To accept 18.0 or 18.4 per cent of sodium sulfate as the concentration which represents the completion of precipitation of casein and pseudoglobulin I tends to introduce an added element of error in the pseudoglobulin II determination. Experiments in which the casein has been removed before precipitation with sodium sulfate indicate that the error for pseudoglobulin II introduced at 18.0 or 18.4 per cent is relatively small.

A third critical zone at 21 to 22 per cent of sodium sulfate is present in colostrum and milk. The difference between the results obtained at 18.4 and 21.5 per cent of sodium sulfate is usually small, it is practically negligible in the case of milk. The addition of saturated sodium sulfate at 37°C. to colostrum precipitates all of the proteins; 30 parts of saturated sodium sulfate to 1 part of colostrum are sufficient.

<sup>3</sup> In the course of experiments dealing with the precipitation of proteins with carbon dioxide it was noted that the addition of potassium oxalate caused the opacity of the solution to disappear, due to the removal of the calcium. A similar phenomenon was observed with sodium citrate (Brown, J. H., and Howe, P. E., *Proc. Bact. Soc.*, 1921); also with sodium sulfate and magnesium sulfate. The addition of calcium chloride causes the opacity to return. This change in appearance has not been commented upon so far as we can find, it must certainly have been observed when citrating milk for infants.

From the results presented and observations made in the course of the work which can be considered as contributory evidence it appears that colostrum may be subdivided by fractionation with sodium sulfate into four protein groups, euglobulin, pseudoglobulin I and casein, pseudoglobulin II, and albumin. Non-protein nitrogen is present in the filtrate from precipitation with saturated sodium sulfate or trichloroacetic acid.

*Determination of Casein in Colostrum.*—In the determination of the proteins of colostrum the estimation of casein presents certain difficulties and at the same time complicates the estimation of the other proteins. Casein is precipitated by concentrations of salts which also precipitate one or both of the pseudoglobulins; saturated magnesium sulfate, ammonium sulfate, precipitation limits 2.2 to 3.6 saturation (13) and sodium sulfate, precipitation limits 16 to 18.4 per cent. On the other hand precipitation with acids, the characteristic property of casein, is a property of euglobulin and saturation with sodium chloride is a property of these two proteins.

In the work of Crowther and Raistrick and of Dudley and Woodman the procedures adopted for the separation of casein, direct precipitation with acetic acid or alum, may or may not succeed according to the quantity of acid or alum added. In case sufficient acid or alum is added to redissolve the euglobulin which is precipitated at low concentrations of these substances separation is essentially complete. The addition of either precipitant to colostrum to the point of ready flocculation does not, however, insure the complete precipitation of casein or the complete solution of euglobulin. A clear supernatant fluid is generally the best evidence of complete precipitation of casein but this has not always been so in our work. The isoelectric point of euglobulin is pH 5.52 (14) and for casein pH 4.4 (15). The point of maximal insolubility has been shown to be at or close to the isoelectric point. From original mixtures of proteins precipitation may occur at a hydron concentration slightly removed from the isoelectric point. Hardy (16) has shown that euglobulin is dissolved at a point at which methyl orange is slightly pink, which represents a hydron concentration of between pH 4 to 5. We can assume, therefore, that recently precipitated euglobulin will be practically dissolved by acid at the isoelectric point of casein. When acidifying with acid to precipitate casein it is desirable to carry the re-

TABLE II—Data Showing the Effect of Acidification and the Addition of Sodium

	Sample A.			Sample B.			Sample C.			Sample D.		
	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.
Total N.....	1.199			2.016			2.365			2.504		
Alum.....	0.578†			0.677†			1.053†			0.875†		
Acetic acid.....	0.573†			0.668†			1.072†			0.866†		
NaCl, saturated....	0.876			1.525			1.717			1.664		
Euglobulin, calculated from NaCl precipitation.....	0.300			0.847			0.651			0.511		
Na <sub>2</sub> SO <sub>4</sub>												
12.5 per cent.....												
13.5 " " .....	0.215	0.358	0.619†	0.592	0.751	0.673†	0.356	0.925	1.084†	0.559	1.084	0.861†
14.0 " " .....	0.254	0.354	0.584†	0.692	0.772	0.657†	0.394	0.909	1.062†	0.853	0.793	0.858†
14.2 " " .....	0.258	0.358	0.576†	0.745	0.593	0.678†	0.416	0.883	1.066†	0.892	0.759	0.853†
14.5 " " .....	0.284	0.345	0.563†	0.865	0.520	0.630†	0.536	0.751	1.078†	1.007	0.644	0.853†
17.4 " " .....	0.778	0.172	0.242	1.581			1.491	0.384	0.490	1.873		
18.4 " " .....	0.893	0.175	0.124	1.700			1.911	0.273	0.171	2.052		
21.5 " " .....	0.991	0.154	0.047	1.773			2.083	0.273	0.009	2.188		
Albumin N + non-protein N...	0.201			0.243			0.282			0.316		
Non-protein N.....				0.081			0.154			0.073		
Euglobulin.....	0.258			0.745			0.416			0.892		
Casein.....	0.576			0.678			1.066			0.853		
Pseudoglobulin I...	0.159			0.277			0.429			0.307		
" II..	0.098			0.073			0.172			0.136		
Albumin.....				0.162			0.128			0.243		

\* Results are expressed as grams of nitrogen in 100 cc. of centrifuged colostrum.

Columns headed "Filtrate N after acidification" refer to nitrogen in the filtrate from the original precipitation. "N precipitated by acetic acid (or alum)" refer to the nitrogen precipitated from the filtrate from the original sodium sulfate precipitation by the addition of acetic acid (or alum).

† Values to be compared with regard to precipitation of casein with acid or alum directly from diluted colostrum and after removal, or partial removal, of euglobulin with sodium sulfate.

*Chloride and Various Concentrations of Sodium Sulfate when Added to Colostrum.\**

Sample E.					Sample F.			Sample G.			Sample H.		
N precipitated.	Filtrate N after neutralization.	N precipitated by acetic acid.	Filtrate N after precipitation with alum.	N precipitated by alum.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.	N precipitated.	Filtrate N after acidification.	N precipitated by acetic acid.
2.819					3.116			3.119			3.213		
1.343†					0.832†			1.110†			0.911†		
0.861†					0.843†			1.110†			1.015†		
2.086								2.031			2.340		
1.305								1.009			1.688		
					0.728	1.092	1.282	0.662	1.403	1.054†			
0.746	1.224	0.849†	1.224	0.849†	1.175	1.092	0.849†	1.020	1.070	1.029†	1.363	1.173	0.677†
0.925	1.113	0.781†	1.105	0.789†	1.184	1.092	0.840†	1.063	1.036	1.020†	1.684	0.934	0.636†
1.129	0.964	0.726†	0.939	0.751†	1.619	0.708	0.789†	1.200	0.897	1.022†	1.776	0.785	0.652†
1.160	0.862	0.797†	0.866	0.793†	1.999	0.529	0.588	1.285	0.853	0.981	2.049	0.691	0.473
2.239	0.316	0.274	0.418	0.162	2.707	0.303	0.106	2.407	0.499	0.213	2.684	0.435	0.094
2.422	0.303	0.094	0.307	0.090	2.711	0.273	0.132				2.736	0.384	0.093
2.474	0.277	0.068	0.273	0.072	2.813	0.196	0.107	2.671	0.119	0.329	2.802	0.260	0.141
0.345					0.303			0.448			0.401		
					0.124			0.171			0.171		
1.129					1.184			1.200			1.776		
0.781					0.840			1.022			0.652		
0.505					0.677						0.308		
0.032					0.102						0.066		
					0.179			0.277			0.230		



action approximately to the isoelectric point of casein. A ready means of doing this is not available since, in addition to the opacity of the colostrum, the indicators which can be used do not show sharp color changes at the desired range of hydron concentrations. Reasonably satisfactory results have, however, been obtained with methyl orange.

The practical difficulty in the separation of casein from the other proteins of colostrum is, then, the means of knowing when sufficient acid or alum has been added to precipitate and redissolve the euglobulin<sup>4</sup> without creating an excess of precipitant which will redissolve the casein. This difficulty is increased in the case of colostrum because of the variable concentration of protein which prevents the possible predetermination of the amount of acid or alum which will produce the desired result such as has been determined for milk. Another factor is introduced when using small amounts of colostrum for analysis in that it is desirable to add the acid or alum in a rather concentrated form in order that the volume of the solution will not be increased appreciably.

As the result of experimentation with the direct precipitation of casein with acetic acid and alum the acceptance of data upon colostrum obtained in this way without other confirmation does not seem justified. The results presented show, however, that the direct precipitation of casein may be and usually is complete. Samples E and H, Table II, are examples of cases in which it was impossible to obtain consistent results by direct precipitation.

The method finally adopted for the determination of casein is to precipitate it from solution by acidification with acid or alum after the euglobulin has been removed with sodium sulfate. The results of representative experiments are contained in Table II. The point to be emphasized with regard to casein from the data presented is that the quantity of protein precipitated with acetic acid, representing casein (similar results have been obtained with alum, Sample E), when added directly to diluted colostrum or to the filtrate from colostrum precipitated at approximately 14.2 per cent of sodium sulfate is essentially the same. These data are indicated by a dagger (†) in the table. The amount of acetic acid

<sup>4</sup> It is possible to add a sufficient excess of either acid or alum such that there will not be a visible precipitation of euglobulin before solution takes place.

which must be added is less than that required for the original colostrum, as a routine procedure 3 drops of 10 per cent acetic acid have been found to be sufficient.

*Procedure for the Analysis of Colostrum.*—The procedures adopted for the analysis of colostrum or milk are based on the considerations discussed above. Material precipitated by 14.2 per cent of sodium sulfate, *i.e.* 14.2 gm. of sodium sulfate contained in 100 cc. of solution, is considered to be euglobulin; that precipitated at 18.4 per cent of sodium sulfate is held to represent euglobulin, pseudoglobulin I, and casein; that at 21.5 per cent of sodium sulfate as precipitating pseudoglobulin II in addition to the proteins given for 18.4 per cent; the remainder of the nitrogen is considered to be albumin and non-protein nitrogen. Casein is determined by acidification of the filtrate from the 14.2 per cent sodium sulfate precipitation. Non-protein nitrogen is determined by precipitation with trichloroacetic acid.

For duplicate precipitations we suggest precipitation of euglobulin at 14.0 and 14.2 per cent of sodium sulfate. If, as sometimes happened, the values so obtained do not agree within reasonable limits, the value at 13.5 usually agrees with that at 14.0 per cent. The duplicate determinations also give two values for casein. As a check on the casein determination a direct precipitation can be made. Precipitations at 17.4 and 18.4 per cent usually agree but not always so, in general the results at 18.4 per cent have agreed with all other procedures. Instead of 21.5 per cent of sodium sulfate a pair of precipitations can be made with 21 and 22 per cent of sodium sulfate. For very viscous samples of colostrum the sample can be weighed out and diluted with 1 per cent sodium sulfate and then weighed amounts of sodium sulfate added to aliquot portions; this procedure requires correction to volume, if the results are to be expressed on the volume basis.

The details of the procedure are as follows: 1.0 cc. portions of centrifuged colostrum are measured into large test-tubes and 30 cc. of the required concentration of sodium sulfate added. The following concentrations of sodium sulfate are prepared, 14, 14.5, 14.65, and 15 per cent sodium sulfate for 13.5, 14.0, 14.2, and 14.5 per cent of sodium sulfate, final concentrations, respectively, when added to colostrum for use at the euglobulin zone; 18 and 19 per cent for 17.4 and 18.4 per cent of sodium sulfate, respectively, at

the end of the pseudoglobulin I precipitation; and 21.7, 22.2, and 22.7 per cent for 21, 21.5, and 22 per cent of sodium sulfate, respectively, for precipitation of the total globulins. 5 per cent trichloroacetic acid is used for the complete precipitation of proteins in the non-protein nitrogen determination. For the determination of casein, 3 drops of 10 per cent acetic acid are added to the filtrate from the sodium sulfate precipitations at the euglobulin critical zone and after the precipitate has settled the solution is filtered and the nitrogen content of the filtrate is determined. The precipitations, with the exception of the trichloroacetic acid precipitation, must be carried out at 34°C., or incubator temperature. The test-tubes are stoppered and permitted to stand until the precipitate has settled, and then filtered. We usually shake up a precipitate after it has settled and allow it to settle once more. Filtrations are conducted into other test-tubes on a dry filter and the funnels are covered with watch-glasses. 5 cc. portions of the filtrates are taken for analysis by the Kjeldahl procedure.

The following calculations can be made:

Total N, determined directly.

Euglobulin N	= Total N - N in filtrate from precipitation with 14.2 per cent sodium sulfate.
Casein N (1)	= N in filtrate from 14.2 per cent sodium sulfate - N in filtrate after acidification.
Casein N (2)	= Total N - N in filtrate after acidification with acetic acid.
Casein N + pseudoglobulin I N	= N in filtrate from precipitation with 14.2 per cent of sodium sulfate - N in filtrate after precipitation with 18.4 per cent sodium sulfate.
Pseudoglobulin I N	= N found for casein + pseudoglobulin I - casein N.
Pseudoglobulin II N	= N in filtrate from precipitation with 18.4 per cent sodium sulfate - N in filtrate from precipitation with 21.5 per cent sodium sulfate.
Albumin N	= N in filtrate from precipitation with 21.5 per cent sodium sulfate - non-protein N.
Non-protein N	= N in filtrate from precipitation with 5 per cent trichloroacetic acid.

It will be noticed that there are but two direct determinations, total nitrogen and non-protein nitrogen; the other determinations depend upon analysis of the filtrate after precipitation and the calculation of the protein precipitated by subtraction. The errors of such methods are appreciated but the procedures outlined seem most nearly to meet the requirements for the differential determination of the proteins of colostrum.

#### DISCUSSION.

Data have been presented which indicate that there are consecutive concentrations of sodium sulfate which, when added to diluted colostrum or milk, do not cause a marked increase in the quantity of protein precipitated and define a critical zone, and that on either side of such a zone a small variation in the quantity of sodium sulfate added results in a relatively large difference in the quantity of protein precipitated. Critical zones are at 14.0 to 14.2, 18.0 to 18.4, and 21 to 22 per cent of sodium sulfate. Similar zones have been obtained in the neutralized filtrates of colostrum from which the casein has been removed with acetic acid. Casein is not precipitated by 14.0 to 14.2 per cent of sodium sulfate but is precipitated by 18.0 to 18.4 per cent sodium sulfate; it is also precipitated by acidification of the diluted colostrum with acetic acid or alum or by the acidification of the filtrate from a precipitation with sodium sulfate. If the sodium sulfate precipitation be made at a concentration less than 14.5 per cent the casein can be recovered completely by subsequent acidification of the filtrate.

As the result of the above observations it is suggested that there exists a basis for the quantitative determination of the various proteins of colostrum or milk. The material separated at the various concentrations of sodium sulfate when added to colostrum in the proportion of 31:1 is considered to consist of the following proteins or mixtures of proteins: (a) at 14.0 to 14.2 per cent of sodium sulfate, euglobulin; (b) at 18.0 to 18.4 per cent of sodium sulfate, euglobulin, pseudoglobulin I, and casein; and (c) at 21 to 22 per cent of sodium sulfate, euglobulin, pseudoglobulin I and II, and casein.

With the values for casein and non-protein nitrogen determined independently we are, then, able to calculate the quantities of the various proteins present in colostrum.

It is necessary to consider the justification for the assignment of names to the proteins of colostrum, with the exception of casein, which are used to designate the proteins of blood.

*Euglobulin.*—The evidence for considering protein precipitated up to and including 14.5 per cent of sodium sulfate in blood as consisting essentially of euglobulin has been presented (11). Similar precipitation limits of a protein in colostrum even though the reaction be slightly different may be taken as presumptive evidence of identity. The following facts tend to support this presumption: (a) precipitation reactions of the purified protein; precipitation by dialysis and by acidification; (b) the proof offered by Crowther and Raistrick, Dudley and Woodman, and Woodman which indicates that the euglobulin of serum and of the colostrum of the cow are identical. These authors support the contention of Chick (17) and Hartley (18) that the protein portion of euglobulin and pseudoglobulin is identical. Chick suggests that euglobulin may be a mechanical complex of a lipoid and pseudoglobulin. The proof with regard to the identity of the protein portion of euglobulin and pseudoglobulin as far as the yield of amino-acids and the rate of racemization are concerned is convincing; we still lack the biological proof, anaphylactic reaction, of the identity of these two fractions such as has been furnished by Wells and Osborne (19) for the individuality of casein, albumin, globulin, and the alcohol-soluble protein of milk. In the work of Wells and Osborne no attempt was made to distinguish between pseudoglobulin and euglobulin of milk, which would be difficult considering the small quantities of globulin present. Osborne (20) did find phosphatide phosphorus present in the globulin fraction. The relatively large percentage of phosphorus present would suggest that the globulin was euglobulin or a mixture of euglobulin and pseudoglobulin. The limited experience which we have had with milk suggests that euglobulin is sometimes present and in other samples of milk it is practically absent.

From the facts in hand we believe that there is sufficient justification for the assumption that the protein precipitated at 14.0 to 14.2 per cent of sodium sulfate consists essentially of a protein, or protein complex, usually designated as euglobulin and that the euglobulin of colostrum is the same as that of blood.

*Pseudoglobulin I.*—The evidence for considering the protein pre-

precipitated between 14.2 and 18.4 per cent of sodium sulfate as consisting essentially of casein and pseudoglobulin I is based upon our results and that of the same group of investigators mentioned above. The distinction between two pseudoglobulins is based largely on our work. Supporting evidence of Crowther and Raistrick, Dudley and Woodman, and Woodman relates to fractions which would include both of the pseudoglobulins. Here as with euglobulin it was found that the pseudoglobulin of colostrum had the same characteristics as that of blood. The question of the identity of pseudoglobulin and euglobulin must be considered here also.

*Casein.*—The presence of casein in the fraction precipitated between 14.2 and 18.4 per cent of sodium sulfate must rest upon results presented in our experimental work and that of Storch; that the material is casein is indicated by the purified product which is precipitated by acids and coagulated by rennin in the presence of added calcium salts.

*Pseudoglobulin II.*—The fraction between 18.4 and 21 to 22 per cent of sodium sulfate does not have any supporting evidence except that presented in connection with this work and the work on blood. That we are dealing with a distinct protein rests on a rather meager basis at present. In colostrum or in blood there is very little of this protein present. The evidence is, however, such that we feel that the fraction should be retained as a probable entity until proof can be presented showing that it is not a particular protein.

Albumin of colostrum and milk has been shown to be chemically different (2, 3, 4) and the albumin of milk to be biologically different (19) from the albumin of blood. The albumin of colostrum and milk should, therefore, be designated lacto-albumin. Data obtained with new-born calves (unpublished) indicate that there is not an increase in the albumin content of the blood at the same time as the increase in the globulins occurs. The increase in albumin which does occur later is a gradual one, suggesting the formation or accumulation of albumin by the calf.

#### SUMMARY.

A basis for the differential estimation of the proteins of colostrum and milk has been presented, and a procedure for the analysis of colostrum suggested.

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# A STUDY OF THE METABOLISM AND RESPIRATORY EXCHANGE IN POULTRY DURING VITAMINE STARVATION AND POLYNEURITIS.

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## INTRODUCTION.

The following investigation was undertaken in order to obtain some information regarding changes that may occur in the metabolism or variations in the respiratory exchange of poultry during vitamine starvation. With the expression vitamine starvation we understand the condition produced by feeding polished rice previous to the appearance of polyneuritis. Chickens were chosen as the experimental animals because they have a high metabolism and in common with other birds they are subject to polyneuritis when fed a diet consisting of polished rice.

We discovered after completing the experimental work described in this paper that Abderhalden (1) had published a series of papers dealing with the effect of a diet of polished rice on pigeons. The above author had measured the carbon dioxide output of the experimental animals and the results are in substantial agreement with our observations on poultry.

## *Methods.*

In studying the respiratory exchange, we used a small respiration apparatus which was an exact duplicate of the one described by Murlin (2).

Careful examination was always made before each experiment to determine whether the apparatus was air-tight. Small leaks very frequently developed and much time was consumed in finding and repairing them. Many experiments were lost for



the reason that leaks occurred during the period of observation. Alcohol checks were frequently made as part of the routine. No animal experiments were included in our results unless we were perfectly certain that the apparatus was tight.

We did not test the residual air in the apparatus for combustible gases. The experiments described by Regnault and Reiset (3) in which chickens were used showed that hydrogen or hydrocarbons generally were not formed or if present the quantity was very small. In determining the respiratory quotient of fattening geese Bleibtreu (4) never found any combustible gases in the apparatus. If any such gases are formed in the digestive tract of chickens the quantity is apparently too small to affect materially our results.

During the periods of observation covered by these experiments the chickens were confined in individual cages in the laboratory. The cages were conveniently arranged for cleaning and for the collection of the excreta. The food was placed before the chickens in separate small containers, arranged so as to prevent food particles being scattered and mixed with the excreta.

The excreta, the mixed feces and urine, were collected in 24 hour periods and dried at a temperature of 60°C. It was then powdered and preserved in glass-stoppered bottles for analysis.

We did not make any temperature observations in our experimental animals. The investigations of Eijkman (5), Abderhalden (6), and Novaro (7) have shown that the temperature of poultry and pigeons always falls a few degrees during vitamine starvation.

#### *Plan of Experiments.*

1. *Normal Basal Metabolism.*—The basal metabolism was determined while the chickens were receiving a ration consisting of grain and mash, which is used in the poultry department of this Station. The experiments in the respiration apparatus were always made in duplicate, the periods usually varying from 60 to 90 minutes each, depending upon the size of the animal. The observations were made from 18 to 20 hours after food had been received. The periods of observation were only begun after the chickens had been in the apparatus from 1 to 1½ hours.

2. *Metabolism during Digestion of Grain and Mash.*—The chickens, having had a liberal quantity of mash before them during

the previous 24 hours, were given in the morning the day's allowance of grain and as soon as this was consumed they were placed in the respiration apparatus. The experiments covered the time from the 2nd to the 6th hour of digestion.

3. *Metabolism during Digestion of Rice.*—These experiments were made in the early part of the rice diet period. The chickens had fasted about 16 hours or from about 4 o'clock in the afternoon until the following morning, when they were allowed to consume as much polished rice as they could eat. Sometimes it was necessary to resort to forcible feeding. The respiratory exchange was determined from the 2nd to the 6th hour of digestion.

4. *Metabolism during Vitamine Starvation.*—A series of experiments was made to determine the respiratory exchange from 18 to 20 hours after feeding rice. Owing to the gradual decline in the intensity of the metabolism, it was generally found necessary during the last 2 weeks of the rice diet to increase the length of the periods of observation to 90 or even 120 minutes each.

This period of exclusive rice diet, which lasted about 6 weeks, we call the period of vitamine starvation. In comparison with the normal basal metabolism it might also be called the basal metabolism on a rice diet. Towards the end and just before polyneuritis became evident, the crop nearly always contained some undigested rice. In fact the period of vitamine starvation cannot be sharply separated from that of polyneuritis. The only way in which we differentiated the two conditions was the appearance of paralysis. When the animal showed evidence of paralysis we classed it as polyneuritic. This is not a strictly true or entirely just definition for the animals undoubtedly had polyneuritis sometime before the disease had progressed so far as to cause paralysis.

5. *Metabolism during Polyneuritis.*—As many experiments as possible were made to determine the gaseous metabolism of the polyneuritic animals. Unfortunately it so happened that the apparatus gave us much trouble at this most critical time and frequently small leaks developed which ruined many of our experimental periods. In some cases we were able to secure only one or two successful experiments.

6. *Metabolism after Recovery from Polyneuritis.*—We were able to secure data regarding the metabolism after recovery from polyneuritis only with Hen 4. This hen had lost nearly 400 gm. in weight during 43 days on the rice diet. The administration of an extract prepared from yeast caused a prompt relief of the symptoms of paralysis in this animal. During the first 2 weeks after recovery the appetite was very poor and only from 15 to 20 gm. of grain were consumed daily. After that time the appetite improved more rapidly but the gain in weight was very slow. At the end of 1 week the hen had gained 20 gm., in 2 weeks 45 gm., in 3 weeks 95 gm., in 4 weeks 160 gm., and in 6 weeks the gain in weight was only 260 gm. In spite of the low food consumption, the metabolism and heat production were practically normal 1 week after recovery as will be noticed by referring to the data regarding Hen 4.

#### *Ration.*

The normal ration consisted of from 20 to 40 gm. each of corn and wheat per day according to the size of the animal. The grain was supplemented by a mash mixture which contained wheat middlings, wheat bran, ground oats, corn-meal, bone meal, blood meal, and meat meal together with a small quantity of sodium chloride. The chickens were allowed as much mash as they could eat but the consumption of this material varied greatly from day to day. Sometimes only a few grams and again more than 50 gm. per day were consumed.

Ample supplies of broken oyster shell and grit and fresh water were always before the animals throughout the experimental period.

During the rice diet ordinary polished rice of the best commercial quality was used.

#### *Effect of Vitamine Starvation and Polyneuritis.*

The first apparent effect on the chickens in vitamine starvation was a loss of appetite, the food consumption falling gradually until the polished rice was entirely refused. Egg production ceased immediately when rice was fed. There was a continuous loss in weight and in about 6 weeks active symptoms of polyneuritis appeared.

While on the rice diet all of the chickens developed what seemed to be more or less severe diarrhea. There were copious liquid discharges which contained much slimy green and white material.

We observed in our experimental animals decided individual variations, not only in regard to the voluntary consumption of polished rice, but in the amount of rice which they were able to digest and assimilate. These variations are indicated in Table I.

When the chickens ceased to consume rice voluntarily they were forcibly fed such quantities of rice as they were able to digest and assimilate during the next 24 hours. The quantity so

TABLE I.  
*Rice Consumption during Vitamine Starvation.*

Hen.....	1	2	3	4	5	6
Number of days when rice was voluntarily consumed.....	3	6	29	27	20	17
Total amount of rice consumed, gm.....	1,530	2,947	1,319	1,525	867	695
Rice consumed per day, gm.....	26	55	30	35	21.6	17
Number of days on exclusive rice diet required for development of complete paralysis.....	44	41	43	43	38	38

fed varied from 75 to 25 gm. per day. If the crop contained much undigested rice on the day following forcible feeding we waited until the crop was nearly empty before additional food was given.

Despite the greatly varying quantities of rice which were consumed by these hens there was very little difference in the time required for the appearance of polyneuritis. Hen 2 consumed  $4\frac{1}{2}$  times more rice than Hen 6, but there were only 3 days difference in time in the development of paralysis. The time required on a diet of polished rice for the appearance of symptoms of paralysis varied from 38 to 44 days. A day or two before paralysis set in there was observed a weakness in the legs and sluggish and uncertain bodily movements. These symptoms increased rapidly in severity and in the course of a day or two the animals became unable to perform any coordinated muscular movements. When disturbed severe convulsions or muscular spasms occurred, particu-

larly in the wings and neck, the head being drawn sharply back between the wings. In this condition the animals presented a strange picture of complete physical incapacity.

All of the chickens lost much weight while on the rice diet and during polyneuritis the weight was reduced to the lowest point. It was somewhat surprising, therefore, on postmortem examination to find that the animals were well covered with fat. In fact the abdominal cavity contained a normal amount of fat. The visceral organs were apparently normal in appearance, the only exceptions noted were that the muscles of the heart, kidneys, and gizzard were slightly softer than in normal birds. The gizzard contained undigested rice, pieces of oyster shell, and gritty particles.

*Respiratory Exchange of Chickens under Normal Conditions in Comparison with Vitamine Starvation and Polyneuritis.*

The average of all the experiments is given in Table II. The data show the carbon dioxide excretion, the oxygen consumption, the respiratory quotient, and heat production of chickens on a normal ration as well as on an exclusive rice diet.

The heat production naturally depended largely upon the muscular activity of the animals during the period of observation. In some experiments the animals were very restless and were almost constantly moving. During some of the experiments the animals were comparatively quiet but at no time were they perfectly quiet throughout the whole period. The degree of muscular activity or restlessness could not be recorded because we were unable to obtain the required pneumograph and other recording appliances.

While none of the chickens was entirely quiet during any experimental period, it must be borne in mind that their muscular activity was much lower than normal. The door of the respiration apparatus was covered by a black cloth and naturally the movements of the chickens in the completely darkened chamber were less pronounced than when they were moving about in their cages in full daylight.

During the basal metabolism the animals were generally restless. The heat production varied by as much as 2 calories per hour in different experiments with the same hen. The value found for

TABLE II.

*Showing Carbon Dioxide Excretion and Oxygen Consumption with the Respiratory Quotient and Heat Production of Chickens under Normal Conditions in Comparison with Vitamine Starvation and Polyneuritis.\**

Hen.....	1	2	3	4	5	6
Normal basal metabolism about 18 hours after food.						
CO <sub>2</sub> , gm. per hr.....	2.697	2.581	1.748	1.615	1.720	1.767
O <sub>2</sub> , gm. per hr.....	2.554	2.428	1.607	1.344	1.476	1.631
R. Q.....	0.77	0.77	0.79	0.87	0.84	0.79
Calories per hr.....	8.38	7.95	5.36	4.59	5.05	5.45
Weight of hen, gm.....	2,610	2,850	1,710	1,700	1,670	1,715
Metabolism during normal digestion of grain and mash.						
CO <sub>2</sub> , gm. per hr.....	3.997	4.741	2.649	2.159	2.345	2.263
O <sub>2</sub> , gm. per hr.....	2.807	3.161	1.865	1.579	1.752	1.632
R. Q.....	1.04	1.09	1.03	0.99	0.97	1.01
Calories per hr.....	9.81	11.15	6.59	5.56	6.14	5.73
Weight of hen, gm.....	2,665	2,960	1,715	1,700	1,797	1,665
Metabolism during digestion of polished rice.						
CO <sub>2</sub> , gm. per hr.....	2.731	3.306	2.266	1.812	2.624	2.536
O <sub>2</sub> , gm. per hr.....	2.076	2.328	1.426	1.252	1.870	1.729
R. Q.....	0.97	1.03	1.18	1.05	1.01	1.07
Calories per hr.....	7.18	8.18	5.01	4.42	6.53	6.10
Weight of hen, gm.....	2,356	2,657	1,716	1,680	1,795	1,685
Metabolism during vitamine starvation.						
CO <sub>2</sub> , gm. per hr.....	1.746	2.222	0.798	0.863	1.052	0.987
O <sub>2</sub> , gm. per hr.....	1.618	1.999	0.791	0.842	1.020	0.949
R. Q.....	0.79	0.82	0.73	0.74	0.75	0.76
Calories per hr.....	5.36	6.68	2.61	2.78	3.38	3.15
Weight of hen, gm.....	2,221	2,530	1,465	1,377	1,451	1,265
Metabolism during polyneuritis.						
CO <sub>2</sub> , gm. per hr.....	1.267	1.782	0.831	1.010	0.856	0.856
O <sub>2</sub> , gm. per hr.....	1.232	1.773	0.848	0.978	0.808	0.804
R. Q.....	0.75	0.73	0.71	0.75	0.77	0.77
Calories per hr.....	4.07	5.81	2.79	3.24	2.69	2.67
Weight of hen, gm.....	1,944	2,330	1,397	1,305	1,256	1,121

\*The figures represent the average value of all experiments.

the basal heat production is, therefore, considerably higher than the true resting metabolism.

It was noticed during the digestion of grain and rice that the animals were more quiet as a rule than when the basal metabolism was determined.

During vitamine starvation Hens 2, 5, and 6 were more restless than the others and this condition is reflected in their higher heat production.

As has been mentioned previously the animals were subject to frequent convulsive movements or muscular spasms during polyneuritis. For this reason the heat production was sometimes higher during polyneuritis than in the more quiet periods just before paralysis set in.

Our figures show that there was hardly any difference in the value of the respiratory quotient observed during vitamine starvation as compared with the normal basal metabolism. Our results in this respect agree with those of Jansen and Mangkoe-winoto (8) but they differ from those reported by Ramoino (9).

The abnormally low respiratory quotients obtained by Ramoino with pigeons fed polished rice were probably due to some leak in the apparatus.

The decline in the intensity of the metabolism, due to loss of appetite and consequent reduction in food consumption and the gradually increasing inability to utilize the food, caused a continuous decrease in the heat production. Towards the end of the vitamine starvation period, the heat production in some cases had fallen 50 per cent below that of the basal metabolism. These observations are in agreement with the results published by Abderhalden (10) with respect to pigeons fed on polished rice.

#### *Calculation of the Non-Protein Respiratory Quotient and Heat Production.*

Since it is ordinarily impossible to determine the urinary nitrogen in birds, it is also impossible to calculate the absolute value for the non-protein respiratory quotient. In our experiments with Hens 1 and 2, the total nitrogen in the excreta was determined and the figures obtained were used in calculating the non-protein respiratory quotient. Unfortunately, this calculation involves an error because some of the excreted nitrogen came from the gut.

In the case of Hen 1, this error was small and could only affect the value for the heat production to a very slight degree. With Hen 2, however, this error is greater because after being on the rice diet for a few days this hen began to pick off and swallow her own feathers. Evidently the feathers contained some substance which contributed to the ability of this hen to utilize polished rice because she was able to digest and assimilate about twice as much rice and her nitrogen excretion was, therefore, about two times greater than in Hen 1.

Owing to the greater utilization of the food, this hen lost very much less in weight and the heat production was much higher during vitamine starvation and polyneuritis than in any of the other hens. In spite of the greater food consumption and generally higher metabolism, there was no delay in the onset of polyneuritis.

The heat production of Hens 3, 4, 5, and 6 as given in Tables II and III, is only approximate because we were unable to analyze the excreta of these animals. The approximate heat production was calculated from the amount of oxygen consumed using the heat value per liter of oxygen as given in the Zuntz table (11) for the observed respiratory quotient. The error involved is not great. We found in the case of Hen 1 that the above mentioned error in calculating the heat production was never greater than 0.15 calorie and frequently the difference was about 0.05 calorie per hour. The error may, therefore, amount to from 0.5 to 1.5 per cent or 1.2 to 3.6 calories per 24 hours. This value is small in comparison with the variations in heat production due to different degrees of muscular activity or restlessness in these animals during the various experiments. The figures for the heat production of these hens, while not quite accurate, are included for the sake of comparison.

#### *Metabolism per Kilo per Hour.*

In Table III, the metabolism is calculated to the basis of kilo weight of animal per hour. The values obtained show decided variation but it could hardly be otherwise owing to the varying degrees of restlessness of the animals during different experimental periods. The average of all the experiments, with all the chickens gives the following values:



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During:	Calories per kilo per hr.
Basal metabolism.....	3.00
Digestion of grain and mash.....	3.55
"    "    rice.....	3.14
Vitamine starvation.....	2.27
Polyneuritis.....	2.26

These figures indicate that there is but little difference in the metabolism and heat production during vitamine starvation and polyneuritis. Broadly speaking we may say that the observed heat production of chickens when calculated to the basis of kilo weight sinks from 3 calories during basal metabolism to

TABLE III.

*Showing the Hourly Excretion of Carbon Dioxide, the Oxygen Consumption, and Heat Production Calculated per Kilo Weight of Chicken.*

Hen.....	1	2	3	4	5	6
Normal basal metabolism.						
CO <sub>2</sub> per kg. per hr., gm.....	1.032	0.905	1.022	0.950	1.032	1.032
O <sub>2</sub> per kg. per hr., gm.....	0.978	0.851	0.938	0.790	0.893	0.951
R. Q.....	0.77	0.77	0.79	0.87	0.84	0.79
Calories per kg. per hr.....	3.21	2.78	3.13	2.70	3.03	3.18
Metabolism during digestion of grain and mash.						
CO <sub>2</sub> per kg. per hr., gm.....	1.497	1.601	1.544	1.269	1.304	1.359
O <sub>2</sub> per kg. per hr., gm.....	1.028	1.068	1.086	0.928	0.975	0.980
R. Q.....	1.04	1.09	1.03	0.99	0.97	1.01
Calories per kg. per hr.....	3.66	3.76	3.84	3.26	3.36	3.45
Metabolism during digestion of polished rice.						
CO <sub>2</sub> per kg. per hr., gm.....	1.146	1.238	1.315	1.078	1.462	1.504
O <sub>2</sub> per kg. per hr., gm.....	0.868	0.872	0.829	0.745	1.041	1.026
R. Q.....	0.97	1.03	1.18	1.05	1.01	1.07
Calories per kg. per hr.....	3.03	3.06	2.88	2.63	3.63	3.62
Metabolism during vitamine starvation.						
CO <sub>2</sub> per kg. per hr., gm.....	0.774	0.876	0.544	0.628	0.720	0.781
O <sub>2</sub> per kg. per hr., gm.....	0.716	0.786	0.539	0.612	0.703	0.750
R. Q.....	0.79	0.82	0.73	0.74	0.75	0.76
Calories per kg. per hr.....	2.37	2.63	1.78	2.02	2.33	2.49

TABLE III—*Concluded.*

Hen .....	1	2	3	4	5	6
Metabolism during polyneuritis.						
CO <sub>2</sub> per kg. per hr., gm.....	0.652	0.764	0.594	0.773	0.714	0.762
O <sub>2</sub> per kg. per hr., gm.....	0.634	0.761	0.606	0.750	0.642	0.715
R. Q.....	0.75	0.73	0.71	0.75	0.77	0.77
Calories per kg. per hr.....	2.09	2.49	1.99	2.48	2.13	2.38

about 2.25 calories per kilo per hour during vitamine starvation and polyneuritis. If the chickens had been entirely quiet these figures would be somewhat lower.

*Percentage Loss in Weight and Decline in Heat Production.*

In Table IV we have compiled the average weight and the calories produced per hour of the experimental animals during the normal basal metabolism in comparison with vitamine starvation and polyneuritis. The figures indicate great individual variations in the different birds. During vitamine starvation the loss in

TABLE IV.

*Percentage Loss in Weight and Decline in Heat Production of the Experimental Animals.*

Hen.	Normal basal.		Vitamine starvation.				Polyneuritis.			
	Average.		Average.				Average.			
	Weight.	Calo-ries.	Weight.	Loss.	Calo-ries.	Loss.	Weight.	Loss.	Calo-ries.	Loss.
	gm.		gm.	per cent		per cent	gm.	per cent		per cent
1	2,610	8.38	2,221	15	5.36	36	1,944	25	4.07	51
2	2,850	7.95	2,530	11	6.68	14	2,330	18	5.81	27
3	1,710	5.36	1,465	14	2.61	51	1,397	19	2.79	48
4	1,700	4.59	1,377	19	2.78	39	1,305	23	3.24	29
5	1,670	5.05	1,451	13	3.38	33	1,256	24	2.69	46
6	1,715	5.45	1,265	26	3.15	42	1,121	34	2.67	51

weight varied from 11 to 26 per cent and the fall in the heat production varied from 14 to 51 per cent. In polyneuritis the loss in weight averaged from 18 to 34 per cent below the normal and the heat production had fallen from 27 to 51 per cent below the basal metabolism.

Hen 2, as already indicated, was able to utilize a greater amount of rice, evidently due to some stimulating effect of the feathers which she swallowed. For this reason, her loss in weight and the decline in heat production were much less than in the other birds.

Hen 4 was in a state of almost constant muscular spasms during polyneuritis. Consequently, the heat production rose considerably above that in vitamine starvation.

*Protein Metabolism on an Exclusive Rice Diet.*

The polished rice supplied a sufficient quantity of protein to cover the requirements of the animals. The sparing action of carbohydrate on the protein metabolism probably accounts for the favorable protein balance during the rice diet. The protein intake was low but the amount of protein metabolized was slightly lower. It has been shown in the case of dogs that a vitamine-free diet does not affect the utilization of protein nitrogen in the alimentary tract (12).

The data on the protein metabolism are given in Table V.

TABLE V.

Hen.	Excreted.			Consumed.			
	Number of days.	Nitrogen.	Protein.	Rice.	Protein.		Protein balance.
		gm.	gm.		per cent	gm.	gm.
1	49	13.05	81.56	1,196	8	95.68	+14.12
2	43	27.69	173.06	2,273	8	181.84	+ 8.78

*Potassium and Phosphorus Metabolism on an Exclusive Rice Diet.*

The figures given in Table VI indicate that the requirements for potassium and phosphorus were very nearly supplied by the polished rice. The negative balance is small when we consider that the experimental period covered from 6 to 7 weeks. The excess of excretion over intake occurred during the last week or two of the experiment when the food consumption was extremely low.

The present study throws no new light on the nature of the special dietary deficiency of polished rice. This subject has been

studied by McCollum and Davis (13) on growing rats. Their results are thus summarized by Sherman (14).

"Polished rice as a diet for growth was found to be deficient in four respects: (1) Its protein content seemed too low for maximum growth, (2) it contained inorganic elements in insufficient amounts and also not in proper proportions, (3) it was found deficient in fat soluble A, and (4) it lacked water soluble B."

The above authors found no evidence of any toxic principle in polished rice as suggested by Caspari and Moszkowski (15).

As shown in our experiments, polyneuritis developed in practically the same length of time irrespective of the quantity of rice that was consumed. In fact, the onset of polyneuritis was slightly delayed in those birds which consumed the larger quantity of rice. These results argue against the presence of any specially toxic principle in polished rice.

TABLE VI.

Hen.	Excreted.			Consumed.					Balance.	
	Number of days.	Potas- sium.	Phos- phorus.	Rice.	Potassium.		Phosphorus.		Potas- sium.	Phos- phorus.
		gm.	gm.		per cent	gm.	per cent	gm.	gm.	gm.
1	49	1.0359	0.9113	1, 196	0.07	0.8372	0.096	1.1482	-0.1987	+0.2369
2	43	2.1724	2.7165	2, 273	0.07	1.5911	0.096	2.1821	-0.5813	-0.5344

As indicated in Table V the rice supplied sufficient protein to cover the metabolic requirements of the birds and the data in Table VI show that the important elements of potassium and phosphorus were supplied in ample amounts as long as the animals were able to assimilate a quantity of rice sufficient to cover their food requirements. Evidently, therefore, the principal cause of the dietary deficiency of rice must be sought in the lacking vitamins or vitamines.

It is known that birds may be kept alive and apparently well for a long time on polished rice when a small quantity of yeast or yeast extract is added daily to the diet. The quantities of protein or mineral constituents contained in the small amount of yeast necessary to prevent the development of polyneuritis would appear to be too insignificant to exert any material influence upon the metabolism.

The vitamines contained in the yeast furnishes some substance or substances which enables the animals to digest and assimilate an adequate quantity of rice to supply the bodily needs of energy. When these substances or vitamins are lacking the processes of oxidation connected with the utilization of food suffer a continuous decline until with the appearance of polyneuritis, they practically cease.

*Metabolism after Recovery from Polyneuritis.*

The sudden rise in the heat production after recovery from polyneuritis is an interesting point which is worthy of further study. Hen 4 had lost 23 per cent in weight while on the rice diet and had suffered a reduction of 39 per cent in the heat production during vitamin starvation as compared with the normal basal. These figures are comparable with the results observed in human beings following undernutrition as discussed by Lusk (16).

A single administration of yeast extract not only relieved the symptoms of polyneuritis but initiated an extremely active metabolism. During the first 2 or 3 weeks after recovery, the food consumption was low and there was very little gain in weight. The heat production, however, of the emaciated organism was equal to that of the well nourished animal.

SUMMARY:

1. The first apparent effect on poultry of a diet of polished rice is a loss of appetite, and food consumption decreases until finally the food is refused entirely. Loss in weight is gradual but continuous and a similar decline in heat production occurs.

2. The fall in the intensity of the metabolism is coincident with the decrease in food consumption; but it depends upon some factors outside of mere voluntary consumption of food, because if the animals are forcibly fed, the utilization of such food is much delayed.

3. The continued lack in the diet of vitamin B evidently causes a serious impairment of the digestive functions which, during polyneuritis, results in a practical cessation of digestion and assimilation.

4. In vitamin starvation there is no noticeable change in the respiratory quotient. During the first 3 or 4 hours after feeding

rice, quotients approaching or exceeding unity were observed until a short time before polyneuritis developed. About 18 hours after feeding rice the average respiratory quotients ranged from 0.73 to 0.82.

5. The most striking effect in vitamine starvation is the inability of the animals to utilize a normal quantity of food and the consequent decided decline in heat production. In some cases the heat production fell from 40 to 50 per cent below the normal basal metabolism.

6. The metabolism sinks to a very low point when polyneuritis has progressed so far that symptoms of paralysis appear. The respiratory quotient seldom rose above 0.75 during this stage of polyneuritis, although the crop contained much undigested rice, indicating an almost complete inability at that time to utilize this food. Undigested rice was found in the crop and gizzard more than a week after the last feeding. The heat production falls to 50 per cent or more below the normal basal metabolism.

7. After the animal recovers from polyneuritis the metabolism and the heat production rise rapidly but the appetite remains poor and the gain in weight is very slow.

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*Hen 1. Rhode Island Red.*

Date.	Experiment No.	Weight.	CO <sub>2</sub> per hour.	O <sub>2</sub> per hour.	R. Q.	Non-protein R. Q.	Nitrogen excreted per day.	Calories per hour.	Duration of experiment.	Condition during experiment.
Normal basal metabolism.										
1921		gm.	gm.	gm.			gm.		min.	
Mar. 18	11	2,550	2.755	2.710	0.74	0.73	1.579	8.86	120	Restless.
" 21	12	2,600	2.585	2.135	0.88	0.91	1.708	7.19	120	Quiet.
" 23	13	2,650	2.815	2.810	0.73	0.71	1.515	9.15	120	Restless.
" 25	14	2,575	2.590	2.450	0.77	0.76	1.571	8.04	120	Moved.
Apr. 9	19	2,675	2.740	2.665	0.75	0.72	1.348	8.69	120	Restless.
Metabolism during digestion of grain and mash.										
Mar. 31	15	2,600	3.780	2.870	0.96	1.00	2.028	9.86	60	Moved.
Apr. 2	16	2,700	4.370	2.760	1.15	1.24	1.619	9.84	120	"
" 5	17	2,725	4.358	2.668	1.19	1.28	1.436	9.55	300	"
" 13	20	2,650	3.758	2.818	0.97	1.00	1.438	9.77	300	"
" 15	21	2,650	3.718	2.920	0.93	0.94	0.876	10.06	300	"
Metabolism during digestion of rice.										
Apr. 20	22	2,600	3.147	2.460	0.93	0.96	1.281	8.43	180	Moved.
" 25	24	2,400	3.130	2.455	0.93	0.94	0.791	8.46	120	"
June 8	31	2,070	1.915	1.315	1.05	1.07	0.220	4.65	180	"
Metabolism during vitamine starvation.										
Apr. 22	23	2,450	2.500	2.350	0.77	0.77	0.5357	7.81	120	Moved.
" 29	25	2,420	2.213	2.047	0.79	0.78	0.3223	6.82	90	"
May 21	27	2,150	1.291	1.181	0.80	0.79	0.2911	3.94	120	Quiet.
June 1	29	2,035	1.423	1.261	0.82	0.82	0.2106	4.24	220	Moved.
" 3	30	2,050	1.305	1.252	0.79	0.79	0.2203	4.02	150	Quiet.
Metabolism during polyneuritis.										
June 14	32	1,980	1.149	1.163	0.72	0.71	0.1621	3.81	180	Cramps.
" 15	33	1,960	1.174	1.123	0.76	0.76	0.1621	3.72	180	"
" 16	34	1,940	1.227	1.209	0.74	0.73	0.1621	3.99	180	"
" 17	35	1,930	1.521	1.502	0.74	0.73	0.1621	4.94	180	Convulsions.
" 18	36	1,910	1.264	1.163	0.79	0.79	0.1621	3.89	185	"

The normal ration consisted of wheat and corn, 40 gm. each, and from 16 to 56 gm. of the mash mixture per day. On Apr. 20 the grain was replaced by polished rice. The exclusive rice diet began Apr. 29. The total amount of rice consumed was 1,530 gm. Paralysis began June 14 and the hen died June 19.

*Hen 2. Rhode Island Red.*

Date.	Experiment No.	Weight.	CO <sub>2</sub> per hour.	O <sub>2</sub> per hour.	R. Q.	Non-protein R. Q.	Nitrogen excreted per day.	Calories per hour.	Duration of experiment.	Condition during experiment.
Normal basal metabolism.										
1921		gm.	gm.	gm.			gm.		min.	
Mar. 17	3	2,750	2.520	2.360	0.78	0.76	1.920	7.73	120	Moved.
" 19	4	2,800	2.580	2.310	0.81	0.82	1.902	7.64	120	"
" 22	5	2,900	2.750	2.700	0.74	0.71	2.238	8.75	60	Restless.
" 24	6	2,900	2.340	2.240	0.75	0.74	2.243	7.28	60	Quiet.
Apr. 1	8	2,900	2.717	2.530	0.78	0.78	1.286	8.36	240	Restless.
Metabolism during digestion of grain and mash.										
Mar. 29	7	3,000	4.344	2.811	1.12	1.21	1.759	9.95	121	Quiet.
Apr. 4	9	3,000	4.894	3.198	1.11	1.22	2.088	11.28	300	Restless.
" 8	11	2,950	5.050	3.210	1.14	1.22	1.766	11.42	60	"
" 12	12	2,950	4.948	3.444	1.04	1.10	1.983	12.07	300	"
" 14	13	2,900	4.472	3.143	1.03	1.07	1.243	11.04	313	Moved.
Metabolism during digestion of rice.										
Apr. 19	14	2,750	4.047	2.942	0.99	1.01	0.892	10.33	240	Restless.
" 23	16	2,700	3.695	2.430	1.11	1.14	0.638	8.61	120	Moved.
" 26	17	2,650	2.915	2.085	1.02	1.05	0.950	7.32	120	Quiet.
June 2	22	2,530	2.567	1.857	1.01	1.03	0.662	6.49	120	"
Metabolism during vitamine starvation.										
Apr. 21	15	2,600	2.330	2.345	0.72	0.71	0.6786	7.68	120	Moved.
" 30	18	2,630	2.605	2.538	0.75	0.74	0.6127	8.37	120	Restless.
May 19	20	2,485	2.288	1.880	0.89	0.90	0.7171	6.40	60	Moved.
" 31	21	2,500	1.989	1.627	0.89	0.90	0.6624	5.53	242	"
June 7	23	2,435	1.899	1.607	0.86	0.87	0.5294	5.44	240	"
Metabolism during polyneuritis.										
June 9	24	2,330	1.782	1.773	0.73	0.72	0.5294	5.81	60	Convulsions.

The normal ration consisted of wheat and corn, 40 gm. each, and the amount of mash mixture consumed varied from 18 to 52 gm. daily. On Apr. 19 the grain was replaced by polished rice. The total amount of rice consumed was 2,947 gm. This hen picked off and swallowed her own feathers while on the rice diet. Paralysis began on June 9 and the hen died June 11.



*Hen 3. White Leghorn.*

Date.	Experiment No.	Weight.	CO <sub>2</sub> per hour.	O <sub>2</sub> per hour.	R. Q.	Estimated calories per hour.	Duration of experiment.	Condition during experiment.
Normal basal metabolism.								
<i>1921</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>			<i>min.</i>	
June 2	1	1,700	1.819	1.502	0.88	5.13	180	Moved.
" 20	3	1,720	1.677	1.711	0.71	5.60	90	"
Metabolism during digestion of grain and mash.								
June 15	2	1,715	2.649	1.865	1.03	6.59	121	Moved.
Metabolism during digestion of rice.								
June 22	4	1,750	2.899	1.700	1.24	6.00	120	Moved.
" 29	5	1,670	1.723	1.318	0.95	4.59	150	"
July 5	6	1,730	2.176	1.261	1.25	4.45	150	"
Metabolism during vitamin starvation.								
July 25	7	1,465	0.798	0.791	0.73	2.61	90	Quiet.
Metabolism during polyneuritis.								
July 28	8	1,420	0.862	0.867	0.72	2.86	180	Restless.
" 30	9	1,375	0.800	0.830	0.70	2.72	180	"

The normal ration consisted of wheat and corn, 30 gm. each, and from 5 to 45 gm. of the mash mixture were consumed per day. The exclusive rice diet began on June 20. The total amount of rice consumed was 1,319 gm. Paralysis began on July 28 and the hen died on Aug. 3.

*Hen 4. White Leghorn.*

Date.	Experiment No.	Weight.	CO <sub>2</sub> per hour.	O <sub>2</sub> per hour.	R. Q.	Estimated calories per hour.	Duration of experiment.	Condition during experiment.
Normal basal metabolism.								
1921		gm.	gm.	gm.			min.	
June 8	1	1,700	1.615	1.344	0.87	4.59	151	Moved.
Metabolism during digestion of grain and mash.								
June 16	2	1,700	2.371	1.735	0.99	6.11	120	Moved.
" 21	3	1,700	1.947	1.424	0.99	5.01	180	Quiet.
Metabolism during digestion of rice.								
June 23	4	1,690	1.898	1.326	1.04	4.68	150	Moved.
" 30	5	1,670	1.726	1.179	1.06	4.16	121	Quiet.
Metabolism during vitamine starvation.								
July 26	6	1,410	0.846	0.808	0.76	2.68	90	Quiet.
" 28	7	1,370	0.817	0.842	0.71	2.76	165	Moved.
Aug. 1	8	1,350	0.928	0.877	0.77	2.92	150	Restless.
Metabolism during polyneuritis.								
Aug. 3	9	1,305	1.010	0.978	0.75	3.24	150	Convulsions.
Metabolism after recovery from polyneuritis.								
Aug. 11	10	1,325	1.570	1.504	0.76	4.99	150	Restless.
" 18	11	1,350	1.751	1.491	0.85	5.07	130	"
Sept. 2	12	1,465	1.827	1.288	1.03	4.51	120	Quiet.

The normal ration consisted of wheat and corn, 30 gm. each, and from 5 to 30 gm. of the mash mixture were consumed daily. The exclusive rice diet began June 21. The total amount of rice consumed was 1,525 gm. Paralysis began Aug. 2. On Aug. 3, the hen received an extract of yeast which relieved the paralysis and complete recovery resulted. The hen weighed 1,565 gm. and was apparently normal when the experiment was concluded on Sept. 14.

*Hen 5. White Leghorn.*

Date.	Experiment No.	Weight.	CO <sub>2</sub> per hour.	O <sub>2</sub> per hour.	R. Q.	Estimated calories per hour.	Duration of experiment.	Condition during experiment
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## Normal basal metabolism.

1921		gm.	gm.	gm.			min.	
June 24	1	1,570	1.688	1.456	0.83	5.04	135	Restless.
July 1	2	1,680	1.719	1.432	0.87	4.89	125	Moved.
Aug. 4	4	1,760	1.755	1.546	0.83	5.23	150	Restless.

## Metabolism during digestion of grain and mash.

July 27	3	1,775	2.263	1.729	0.95	6.03	150	Moved.
Aug. 5	5	1,820	2.427	1.776	0.99	6.26	120	"

## Metabolism during digestion of rice.

Aug. 9	6	1,790	2.976	1.920	1.12	6.78	135	Restless.
" 13	7	1,800	2.272	1.820	0.91	6.28	120	"

## Metabolism during vitamine starvation.

Aug. 29	8	1,500	0.974	0.912	0.78	3.04	150	Quiet.
" 31	9	1,450	1.099	1.126	0.71	3.69	120	Restless.
Sept. 3	10	1,405	1.084	1.022	0.77	3.40	120	Moved.

## Metabolism during polyneuritis.

Sept. 7	11	1,280	0.872	0.824	0.77	2.74	75	Quiet.
" 9	12	1,265	0.935	0.876	0.77	2.92	150	Moved.
" 12	13	1,225	0.763	0.723	0.77	2.41	180	Quiet.

The normal ration consisted of wheat and corn, from 20 to 25 gm. each, and from 5 to 45 gm. of the mash mixture were consumed daily. The exclusive rice diet began Aug. 6. The total amount of rice consumed was 867 gm. The first signs of polyneuritis began Sept. 7 and paralysis set in Sept. 12. An extract of yeast was given in the afternoon of Sept. 12, but there was no improvement and the hen died Sept. 14.

*Hen 6. White Leghorn.*

Date.	Experiment No.	Weight.	CO <sub>2</sub> per hour.	O <sub>2</sub> per hour.	R. Q.	Estimated calories per hour.	Duration of experiment.	Condition during experiment.
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## Normal basal metabolism.

1921		gm.	gm.	gm.			min.	
June 25	1	1,725	1.703	1.639	0.76	5.43	120	Restless.
July 29	3	1,715	1.700	1.485	0.83	5.02	150	"
Aug. 5	4	1,705	1.899	1.768	0.78	5.91	120	"

## Metabolism during digestion of grain and mash.

July 2	2	1,670	2.026	1.529	0.96	5.34	123	Moved.
Aug. 6	5	1,660	2.501	1.736	1.06	6.13	120	"

## Metabolism during digestion of rice.

Aug. 10	6	1,680	2.642	1.816	1.06	6.41	135	Restless.
" 12	7	1,690	2.430	1.643	1.07	5.80	120	Moved.

## Metabolism during vitamine starvation.

Aug. 30	8	1,305	0.938	0.926	0.74	3.06	150	Quiet.
Sept. 1	9	1,280	1.050	0.991	0.77	3.30	150	Moved.
" 6	10	1,210	0.974	0.930	0.76	3.09	205	"

## Metabolism during polyneuritis.

Sept. 8	11	1,150	0.918	0.832	0.80	2.79	86	Quiet.
" 10	12	1,140	0.890	0.859	0.75	2.84	165	Cramps.
" 13	13	1,075	0.760	0.720	0.77	2.40	90	Quiet.

The normal ration consisted of wheat and corn, from 20 to 25 gm. each, and from 5 to 45 gm. of the mash mixture were consumed daily. The exclusive rice diet began Aug. 6. The total amount of rice consumed was 695 gm. The first signs of polyneuritis began on Aug. 8 and paralysis set in Sept. 12. An extract of yeast was given Sept. 13 and the hen recovered.



## NITROGEN NUTRITION OF YEAST.\*

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A review of the literature on yeast growth and on the nitrogen nutrition of yeast leads one to the conclusions that ammonium salts, proteins, amino-acids, or acid amides may be used as the source of nitrogen and that the rate of growth as well as the composition of the yeast is influenced not only by the character of the nitrogen source, but also very decidedly by the concentration thereof within rather narrow limits. We also conclude that other factors are very essential in the growth of yeast. These other factors are the true nutrients, carbohydrates, inorganic salts, and water, also aeration and temperature, and finally the growth stimulating vitamine or "bios" of Wildier (1 to 26).

While on the whole these general conclusions may be correct, nevertheless one is struck by the fact that the last named factor has in no case been properly controlled in a study of the nitrogen nutrition of yeast. It was therefore considered advisable to carry out a systematic study of the nitrogen content and yield of yeast under conditions where the vitamine factor can be controlled. Accordingly, studies were carried out with a constant concentration of vitamine in media containing as nitrogen sources, variously hydrolyzed protein with and without the addition of certain amino-acids or several other nitrogenous compounds in various concentrations.

### *Methods Used.*

#### *The Method of Growing Yeast.*

About 0.15 gm. of compressed yeast is suspended in 1 liter of sterilized distilled water and shaken so as to obtain a slightly cloudy liquid. The heavier clumps of yeast cells are allowed to

\* This work was carried out in connection with a fellowship for the study of yeast nutrition given by the Fleischmann Co.

settle out and thus a fairly uniform suspension, consisting mostly of single cells is obtained at the surface; 1 cc. of such a suspension contains about 75,000 to 100,000 yeast cells. As the number of cells is, of course, constant for only the one set of experiments in which the same yeast suspension is used, the proper control has to be made in each case so as to obtain a basis for comparing the results from seedings of the different suspensions.

1 cc. of the yeast suspension is drawn off from the surface by means of a pipette and introduced into 125 cc. of the medium, contained in a 300 cc. Erlenmeyer flask and previously sterilized at 10 pounds pressure for 10 minutes. As 300 cc. Erlenmeyer flasks are used throughout the experiments, the surface exposed to the air is practically the same in each case. The yeast is grown at 30°C. for 20 hours. This time was chosen because in this stage practically no decomposition of cells takes place, but growth only. As we were not interested to follow fermentation as such, but the effect of various nitrogenous substances on growth, a point of the growth curve of Rubner was chosen which was not optimal, but nevertheless sufficiently high, so as to have the largest possible growth with the least decomposition of yeast cells.

After the growth period of 20 hours a few drops of formaldehyde are added, so as to stop the growth. In those cases where the amino nitrogen was estimated in the filtrate from the yeast the addition of formaldehyde was omitted. The yeast is then rapidly filtered off into weighed Gooch crucibles. A layer of asbestos of such thickness that the holes of the crucible can scarcely be noticed when held against the light, is very satisfactory. The yeast is washed with a uniform amount of water, so as to avoid variations due to the solvent action of water. About two Gooch crucibles full of water are sufficient to remove completely the unfermented sugar of the medium. The yeast is dried in an oven for about 40 minutes at 103°C. and, when sufficiently cooled in a desiccator, is weighed accurately. The asbestos with the yeast layer is transferred to a Pyrex test-tube of large diameter and the nitrogen content determined as outlined below.

#### *The Method for Micro Kjeldahl Nitrogen.*

The digestion is carried out for 3 hours using 1.5 cc. of concentrated sulfuric acid, 1 gm. of potassium sulfate, and 1 drop of the

5 per cent copper sulfate solution. To prevent loss by spattering and by boiling to complete dryness, glass boiling caps are used. After complete digestion and cooling, the residue is diluted with 15 cc. of ammonia-free water and after the addition of 4.5 cc. of saturated sodium hydroxide the ammonia is distilled into a 100

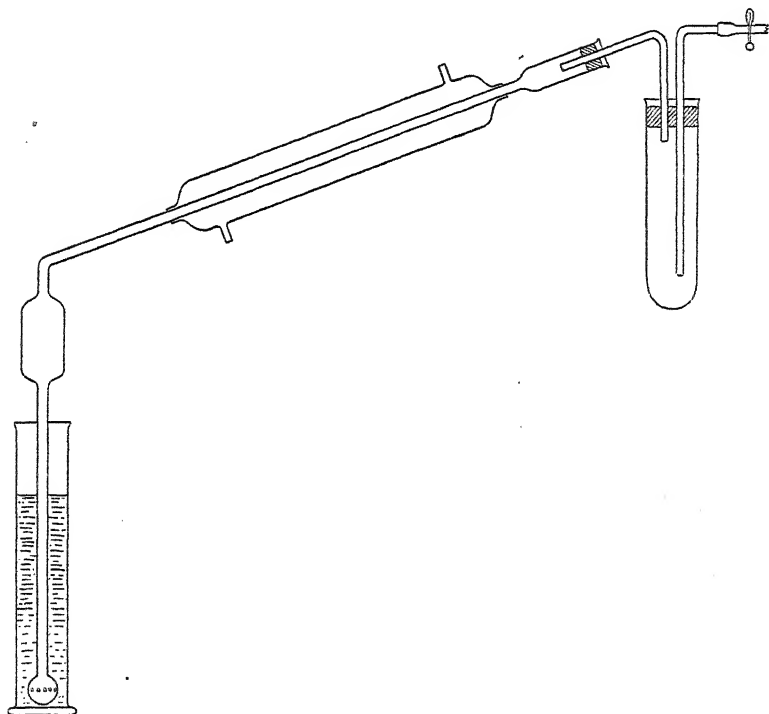


FIG. 1.

cc. graduated cylinder containing 75 cc. of an approximately 0.005 N HCl solution. The apparatus as used is shown in Fig. 1. Without this long absorbing column of acid and the Folin bulb with the small openings for breaking the gases into small bubbles there is appreciable loss of ammonia. It is also wise to add the same concentration of HCl to the standard  $(\text{NH}_4)_2\text{SO}_4$  before Nesslerizing, because even this low concentration of chloride ion is sufficient to appreciably decrease the color. Nesslerization is conducted in the usual manner.



*The Preparation of the Vitamine Solution.*

9 pounds of Fleischmann's starch-free yeast were extracted in the water bath three times with 90 per cent alcohol. The extracts were filtered off hot. On cooling, fatty material, phytosterol, and small quantities of a prolamine separated. The extracts were allowed to stand in the refrigerator for several days. The clear liquid was decanted and the residue was washed with alcohol. The washings and combined extracts were evaporated on the water bath to a syrupy mass and this then taken up with water. The insoluble residue was filtered off and discarded. The water solution after concentrating and shaking out with ether, to remove fatty materials, was then evaporated to dryness under diminished pressure and extracted three times with 600 cc. of 90 per cent boiling alcohol. The insoluble residue, when examined by the micro yeast vitamine method as published in a previous paper, was found to be very low in vitamine content as compared with these alcoholic extracts. On standing in the ice box a needle-like substance separated from the concentrated extracts. This was found to be inosite. After the removal of these crystals, which were found to be inactive from the growth-promoting standpoint, the alcoholic solution was made up to a definite volume.

From the standard alcoholic vitamine solution a vitamine solution, V. S. II, was prepared by evaporating 25 cc. of the stock solution to dryness on the water bath and taking up the residue in water and diluting to 200 cc. This solution was analyzed for various constituents with the results indicated below:

The values given are for 3 cc. of the solution, V. S. II, because this is the amount of vitamine solution used in each flask throughout the work.

3 cc. of solution, V. S. II, contained:

	N	Total.
	<i>mg.</i>	<i>per cent</i>
Total N.....	2.76	100
Amino N.....	0.74	26.8
Amide N.....	0.09	3.4
Ammonia N.....	0.05	1.6
Undetermined N.....	1.88	68.2
Total solids.....	33.3	100
Inorganic solids.....	3.39	10.2
Organic solids.....	29.9	89.8

The above figures show that the preparation is mainly organic in nature with the greater part of nitrogen in an undetermined form.

The total nitrogen was determined by the micro Kjeldahl method as indicated above. The amino nitrogen was determined by the Van Slyke method. The ammonia nitrogen by the permutit method. The amide nitrogen was determined by a combination of the usual procedure of hydrolysis with 5 per cent  $H_2SO_4$  and determination of the resulting ammonia by the permutit process. This method was checked on a standard solution of asparagine before it was applied. It was found to check within less than 1 per cent error.

### *The Preparation of Edestin.*

Edestin was prepared by the sodium benzoate method of G. Reeves. It was recrystallized from a sodium chloride solution, so as to remove any traces of the sodium benzoate, washed thoroughly with  $CO_2$ -free water, then once with 50 per cent alcohol, using the centrifuging method for the washing and thus avoiding the action of air. The thick paste was then rapidly thrown on a Buchner filter and washed with absolute alcohol and ether. The product obtained was white in color and almost entirely soluble in 10 per cent NaCl.

A portion of the edestin was extracted with alcohol and analyzed for vitamine by the micro yeast method described in a previous paper. No vitamine was found present.

The edestin hydrolyzed for different lengths of time also was found deficient in the yeast growth-stimulating vitamine.

### *Media Used.*

From time to time various media were used. The compositions, thereof are given below. All the inorganic salts used were of the "analyzed" grade, the cane-sugar was either Kahlbaum's best crystalline or domestic rock-candy. The asparagine was recrystallized from water two or three times.

Medium .....	1	2	3	4
	gm.	gm.	gm.	gm.
Cane-sugar.....	20	20	20	100
Ammonium sulfate.....	3		3	
Asparagine.....	.0		1.5	
KH <sub>2</sub> PO <sub>4</sub> .....	2	2	2.0	10
MgSO <sub>4</sub> .....	0.25	0.25	0.25	1.25
CaCl <sub>2</sub> .....	0.25	0.25	0.25	1.25
Vitamine solution (V. S. II) ..				17.8 cc.
Diluted to.....	1,000 cc.	1,000 cc.	1,000 cc.	500 cc.

## EXPERIMENTAL.

*Determination of the Concentration of the Vitamine Solution To Be Used.*

A series of 300 cc. Erlenmeyer flasks was prepared containing increasing amounts (0, 3, 4, and 10 cc. portions, respectively) of the vitamine solution, V. S. II, together with 100 cc. of Medium 3. Otherwise the usual method for the growth test was followed. Table I indicates in detail how many trials were made. In all the subsequent tables the duplicate values will not be given, but the results recorded are in each case averages of duplicate tests on growth of yeast and the nitrogen content thereof.

TABLE I.

Vitamine solution	Dried yeast.	N in the dry yeast.	N absorbed from medium.
cc.	mg.	per cent	per cent
0	2.6	5.2	0.15
0	3.0	4.5	0.15
3	34.8	10.1	3.9
3	38.9	10.1	4.3
4	40.4	9.6	4.2
4	44.6	10.4	5.0
10	42.2	10.3	4.6
10	41.3	10.4	4.6

The results show close agreement in the duplicates and that 3 cc. of vitamine solution are ample to bring about a good growth in 20 hours. It is also evident that the yeast grown without vitamine is very low in nitrogen in spite of the same amount of

available asparagine nitrogen. Increasing the amount of vitamin solution above 3 cc. does not appear to improve the nitrogen absorption of the growing yeast.

*Asparagine as a Source of Nitrogen for Yeast.*

Asparagine is an excellent source for yeast nitrogen. The question is, which nitrogen in the asparagine molecule is the active one. To study this question two lines of experimentation were undertaken: (a) The effect of various substances closely related to asparagine was studied and (b), the asparagine-containing medium in which yeast had been grown was investigated in order to determine if possible what had become of the asparagine utilized.

TABLE II.

Substance added.	In Medium 1 in presence of $(\text{NH}_4)_2\text{SO}_4$ .			In Medium 2 in the absence of $(\text{NH}_4)_2\text{SO}_4$ .		
	Dried yeast.	N therein.	N absorbed from medium.	Dried yeast.	N therein.	N absorbed.
	mg.	per cent	per cent	mg.	per cent	per cent
Succinimide.....	13.5	9.8	1.6	9.5	6.1	3.0
Succinamide.....	23.1	7.9	1.8	7.9	8.5	1.7
Aspartic acid.....	17.0	10.7	2.2	11.7	11.6	7.3
Asparagine.....	36.8	10.1	3.8	15.9	9.7	5.0
Vitamine only.....	27.5	7.1	3.0	8.5	6.4	19.7

In the former studies 0.15 gm. of the substance, asparagine, succinimide, succinamide, and aspartic acid, respectively, was added as usual to 100 cc. of Medium 1 together with the 3 cc. of vitamin solution and compared with the proper control, and again, the same substances were used in the same concentration under comparable conditions in Medium 2. Thus the effect of these substances was studied upon the growth of yeast in the presence and in the absence of ammonium sulfate. Table II gives a summary of the results obtained.

In the second series the distribution of nitrogen between the different forms was investigated in the original medium and in the filtrate from the yeast growth. The results are given in Table III.

TABLE III.

*Summation of Results Obtained on Nitrogen Distribution in Asparagine Solution in the Absence of  $(\text{NH}_4)_2\text{SO}_4$ , after Growth of Yeast Therein.*

Distribution of N.	Asparagine-free medium.	Asparagine-containing medium.
	mg.	mg.
Original medium.		
Total amide N.....	0.09	13.74
" amino N.....	0.74	14.54
" $\text{NH}_3\text{N}$ .....	0.05	0.05
" N.....	2.76	30.21
Yeast filtered off.		
Weight of yeast.....	26.85	48.7
N in the yeast.....	1.8	4.88
Filtrate from the yeast.		
Total amide N.....	0	13.21
" amino N.....	0	11.75
" $\text{NH}_3\text{N}$ .....	0.22	0.22
" N.....	0.22	25.18
Sum of the total N in filtrate and in yeast.....	2.02	30.06

*Hydrolyzed Edestin as a Source of Nitrogen.*

Edestin was hydrolyzed for varying lengths of time with 25 per cent  $\text{H}_2\text{SO}_4$ , 5 per cent  $\text{H}_2\text{SO}_4$ , saturated  $\text{Ba}(\text{OH})_2$ , and by both  $\text{Ba}(\text{OH})_2$  and  $\text{H}_2\text{SO}_4$ , respectively, at boiling temperature. In each case 1 gm. of the protein was employed and after the close of the period of hydrolysis the  $\text{SO}_4$  and Ba were removed so as to leave only a trace of  $\text{H}_2\text{SO}_4$  in the solution. Of such solutions the equivalent of 0.1 gm. of original edestin was added to 10 cc. of the special Medium 4, and the whole made up to 125 cc. The solution thus obtained contained the same concentration of cane-sugar, etc. as Medium 2. Such solutions were then sterilized in an autoclave and used for a 20 hour growth test in the usual manner.

*Action of Edestin Hydrolyzed for 5 and 27 Hours, Respectively, in 25 Per Cent  $\text{H}_2\text{SO}_4$ .—*Two 1 gm. portions of edestin were boiled with 10 cc. of 25 per cent  $\text{H}_2\text{SO}_4$  under a reflux condenser for 5 and 27 hours, respectively. After removal of the  $\text{H}_2\text{SO}_4$  by

$\text{Ba}(\text{OH})_2$  to the point where only very little  $\text{H}_2\text{SO}_4$  remained and then concentrating to a definite volume the two hydrolysates were examined for total nitrogen and amino nitrogen, and for yeast growth with the addition of vitamine. The total and amino nitrogen contents were the same in both hydrolysates; viz., 135 to 136 mg. of total nitrogen and 72.2 to 72.7 mg. of amino nitrogen per 1 gm. of original edestin. These results indicate a complete hydrolysis and no change after 5 hours boiling. The yeast growth experiments, however, show that the 5 hour hydrolysis is much more efficient as a nitrogen food than the 27 hour hydrolysis. This is shown in Section A of Table IV.

*Action of Edestin Hydrolyzed by 5 Per Cent  $\text{H}_2\text{SO}_4$ .*—Following the same procedure as above, 1 gm. portions of edestin were hydrolyzed with 10 cc. of 5 per cent  $\text{H}_2\text{SO}_4$  for  $8\frac{1}{2}$ , 18,  $37\frac{1}{2}$ , and 148 hours, respectively. Here also total and amino nitrogen estimation and duplicate growth tests were made. The results are given in Section B, Table IV.

The effects of varying concentrations of the edestin hydrolyzed for 148 hours are shown in Section C, Table IV.

*Action of Edestin Hydrolyzed by Saturated  $\text{Ba}(\text{OH})_2$ .*—1 gm. amounts of edestin were boiled for 24, 75, and 148 hours, respectively, with 10 cc. of  $\text{Ba}(\text{OH})_2$  solution (saturated at room temperature). At the close of the period the Ba was removed by a slight excess of  $\text{H}_2\text{SO}_4$  and after concentrating the solution to a definite volume, studied in the same general way. The results are given under Section D, Table IV.

*Comparative Tests on Acid, Alkaline, and Combined Alkaline and Acid Hydrolyzed Edestin, Respectively.*—The results in the series above indicate that the more prolonged alkaline hydrolysis is not desirable. Does it permanently injure the edestin as a nitrogen source or may a subsequent hydrolysis by acid improve it? To determine this a 148 hour  $\text{Ba}(\text{OH})_2$  hydrolyzed edestin was compared with a 149 hour 5 per cent  $\text{H}_2\text{SO}_4$  hydrolysate as well as with one in which the 148 hour alkaline hydrolysate by saturated  $\text{Ba}(\text{OH})_2$  was followed by a 70 hour hydrolysis with 5 per cent  $\text{H}_2\text{SO}_4$ . The results are given in Section E of Table IV.

TABLE IV.

Section.	Mode of hydrolysis.	N distribution in medium.				Results of yeast growth tests.		
		Edestin N.		Vitamine solution N.		Weight of dry yeast.	N in yeast.	N absorbed from medium.
		Total.	Amino.	Total.	Amino.			
		mg.	mg.	mg.	mg.	mg.	per cent	per cent
A	25 per cent $H_2SO_4$							
	5 hrs.....	13.6	7.27			51.8	10.5	33.2
	27 " .....	13.5	7.22	2.76	0.74	36.6	8.5	19.1
	Control.....	0	0			14.7	6.5	37.3
B	5 per cent $H_2SO_4$							
	8.5 hrs.....	12.56	4.87			59.0	9.3	35.8
	18 " .....	13.15	5.19			50.5	10.4	32.8
	37.5 " .....	13.62	7.13	2.76	0.74	47.2	10.1	29.1
	148 " .....	11.98	6.72			43.1	7.3	21.6
	Control.....	0	0			11.7	5.3	22.7
C	5 per cent $H_2SO_4$							
	148 hrs.....	31.8				26.2	9.8	7.4
	148 " .....	10.5				36.2	8.8	24.1
	148 " .....	8.1		2.76	0.74	44.7	9.9	40.6
	148 " .....	2.1				27.9	8.9	46.3
	Control.....	0	0			10.1	9.0	33.0
D	Ba (OH) $_2$ .....							
	24 hrs.....	11.22	2.5			23.6	7.3	12.3
	75 " .....	11.94	2.2	2.76	0.74	13.5	6.2	5.7
	148 " .....	12.60	2.5			12.1	5.5	4.3
	Control.....	0	0			11.8	5.5	23.5
E	5 per cent $H_2SO_4$							
	148 hrs.....	14.07	8.52			21.2	8.2	10.4
	148 " Ba (OH) $_2$ .....	12.60	2.46			9.7	6.4	4.0
	148 " Ba (OH) $_2$ .....	11.80	6.72	2.76	0.74	27.3	9.3	17.4
	70 " 5 percent $H_2SO_4$ .....	11.80	6.72	2.76	0.74	27.3	9.3	17.4
	Control.....	0	0			11.7	6.9	29.4

*Amino-Acids as a Source of Nitrogen.*

The results obtained with edestin, hydrolyzed in various ways and to various degrees of completeness, indicate that possibly

TABLE V.

*The Effect of Amino-Acids on Yeast Growth in Media Containing a Constant Amount of the Vitamine but with and without Hydrolyzed Edestin, Respectively.*

Amino-acid added.	Amount of acid added.	Hydrolyzed edestin. Added + Not added -	Results of yeast growth tests.		
			Weight of dry yeast.	N in yeast.	N absorbed from medium.
	mg.		mg.	per cent	per cent
Cystine.....	20	+	62.2	8.7	28.3
" .....	10	+	44.5	10.0	24.7
" .....	20	-	7.1	5.2	7.3
" .....	10	-	6.5	6.1	10.0
Control.....	0	+	20.7	8.8	10.9
" .....	0	-	10.2	8.3	30.5
Cystine and tyrosine, each.	20	+	31.3	8.1	12.2
" " " "	10	+	19.8	9.3	9.8
" " " "	20	-	3.8	6.0	3.5
" " " "	10	-	5.9	5.7	7.3
Control.....	0	+	19.0	9.1	10.6
" .....	0	-	10.2	8.3	30.5
Tyrosine.....	20	+	31.4	8.3	14.2
" .....	10	+	43.6	12.1	30.0
" .....	20	-	9.3	7.2	15.6
" .....	10	-	17.1	7.5	36.1
Tryptophane.....	3	+	34.2	9.1	18.0
" .....	1.5	+	34.1	8.6	17.1
" .....	3	-	10.8	5.0	16.9
" .....	1.5	-	10.0	4.7	15.6
Control.....	0	+	27.1	8.6	13.9
" .....	0	-	8.5	4.8	14.9
Glucosamine.....	2	+	21.9	8.3	10.7
" .....	10	+	21.2	8.2	9.7
Control.....	0	+	21.2	8.2	10.3
" .....	0	-	11.7	6.9	29.1
Histidine.....	1.0	+	19.3	9.7	11.3
" .....	0.5	+	24.9	10.1	14.8
" .....	1.0	-	6.1	6.2	13.8
" .....	0.5	-	8.4	6.7	20.5



TABLE V—*Concluded.*

Amino-acid added.	Amount of acid added.	Hydrolyzed edestin. Added + Not added -	Results of yeast growth tests.		
			Weight of dry yeast.	N in yeast.	N absorbed from medium.
	<i>mg.</i>		<i>mg.</i>	<i>per cent</i>	<i>per cent</i>
Proline1.....	4.94	+	24.9	8.6	12.1
“ 1.....	2.47	+	41.0	10.5	25.1
“ 1.....	4.94	—	9.5	3.6	10.4
“ 1.....	2.47	—	9.5	4.5	13.6
Control.....	0	+	24.9	8.6	12.7
“ .....	0	—	8.7	5.1	16.2
Proline2.....	7.5	+	20.8	7.9	9.5
“ 2.....	3.7	+	29.1	7.5	12.8
Glucosamine.....	2.0	+	21.6	8.2	10.5
“ .....	1.0	+	19.4	8.2	9.5
“ .....	2.0	—	7.9	6.6	18.9
“ .....	1.0	—	9.2	6.0	20.1
Control.....	0	+	21.9	8.6	11.3
“ .....	0	—	11.8	5.5	23.5
Lysine.....	2.48	+	24.9	9.6	13.7
“ .....	1.24	+	28.0	7.1	11.6
“ .....	2.48	—	13.9	8.0	34.4
“ .....	1.24	—	13.9	8.2	37.7
Arginine.....	3.6	+	24.8	9.1	12.8
“ .....	1.8	+	26.0	8.6	13.0
“ .....	3.6	—	15.8	8.9	38.7
“ .....	1.8	—	16.1	7.7	38.0
Control.....	0	+	21.3	8.2	10.4
“ .....	0	—	11.7	6.9	29.1

certain specific amino-acids are very important in yeast growth. The results referred to may possibly be in part attributed to the destruction of certain amino-acids in the process of hydrolysis or to the loss of some of them due to precipitation thereof in the neutralization and removal of  $\text{SO}_4$  or Ba. That this may in part be correct is suggested by the results obtained, but we may also attribute the deleterious effect of completely hydrolyzed edestin as in part due to too high a concentration of certain amino-acids in the free form.

In these studies pure amino-acids in quantities varying from 1 to 20 mg. were added to 125 cc. of the usual Medium 2 with

and without the addition of 0.1 gm. of edestin previously hydrolyzed for 148 hours with 5 per cent  $\text{H}_2\text{SO}_4$  and freed from  $\text{SO}_4$  in the usual way. Wherever the hydrolyzed edestin was added it is to be remembered that 14.07 mg. of total edestin nitrogen and 8.52 mg. of amino nitrogen from edestin were present in the usual 125 cc. of medium employed for the growth tests. The usual control tests were made. Table V gives the results of these tests.

In the case of cystine and tyrosine the amount indicated was first dissolved in  $\text{KH}_2\text{PO}_4$  of a known concentration and this was then diluted so that the final medium was of the usual concentration in K,  $\text{PO}_4$ , etc., as in the other tests.

In the case of proline it was prepared according to the Dakin method (27) by extraction with butyl alcohol. The copper salt proved to have 17.8 per cent impurities as determined by total and amino nitrogen estimations. Nevertheless this impure product was tried for our purpose.

As the action observed may have been due to the impurity present in the preparation, a purification of the proline was attempted. By fractional crystallization of the copper salt a product was obtained containing only 6.2 per cent impurities.

#### DISCUSSION.

*The Utilization of Asparagine Nitrogen.*—That asparagine is a particularly good form of nitrogen for yeast growth is well shown by the results in Table II. The yield of yeast is highest in the presence of a constant concentration of asparagine and vitamine no matter whether ammonium sulfate is present or absent. However, in presence of asparagine, the yield of yeast is slightly more than doubled in case ammonium sulfate is present, whereas in the absence of asparagine the ammonium sulfate increases the yield over threefold. Similar results are obtained in the presence of succinamide. Succinimide and aspartic acid, however, increase the yield of yeast only slightly in the absence of  $(\text{NH}_4)_2\text{SO}_4$  and actually decrease it decidedly in the presence of  $(\text{NH}_4)_2\text{SO}_4$  as compared with the control test with vitamine only. However, an ammonium sulphate medium increases the yield of yeast in every case here studied. The results do not show which form of nitrogen is used in the ammonium sulfate-containing medium.

It is to be noted that the nitrogen content of yeast varies considerably and that the most favorable medium for the growth of a high nitrogen yeast is one containing either asparagine or aspartic acid. The results of Table II suggest that the  $\alpha$ -amino group of asparagine or aspartic acid stimulates nitrogen assimilation, but that the amide group also aids in stimulating cell reproduction provided ammonia is present as such or in an available form as in the  $\alpha$ -amino group in asparagine or aspartic acid. Certainly the combination of the amide and  $\alpha$ -amino groups appears very favorable for yeast growth.

This is in part confirmed by the results in Table III. If we assume that the vitamine solution contributed as much of its nitrogen in the case where asparagine was present as in the control test, we must account for 3.08 mg. of yeast nitrogen obtained from the asparagine. Inspection of the table shows that of this nitrogen, 2.05 mg. (66.6 per cent) were  $\alpha$ -amino in character, 0.44 mg. (14.3 per cent) was amide nitrogen, and the remainder (19.1 per cent) not accounted for. The 5.03 mg. of total nitrogen lost from the medium checks fairly well with the 4.88 mg. found in the yeast filtered off. It is also of some interest to note that the ammonia content of the two filtrates is the same even in spite of the difference in yeast growth. It appears then that the asparagine has been deaminized only in so far as the nitrogen has been used for yeast growth or for the synthesis of yeast protein. The amide group in asparagine has been only slightly affected, but the  $\alpha$ -amino group is used up almost quantitatively in proportion to the yeast growth and the deaminized asparagine is left practically intact in the filtrate. The amino nitrogen in the vitamine solution is used up practically completely. Waterman (24) arrived at very similar conclusions. On the whole the results for asparagine agree with Ehrlich's view that amino-acids are deaminized before the nitrogen is used for growth and that they are not utilized as such (22).

*Hydrolyzed Edestin as a Source of Yeast Nitrogen.*—The first striking conclusions inferred from Section A, Table IV are that although the free amino nitrogen estimation indicates the same stage of hydrolysis of edestin in the 5 and 27 hour hydrolysates, still the shorter period yields a medium much more efficient as a yeast nutrient both from the standpoint of yield of

yeast as well as from the nitrogen content of the dry yeast. If we use a weaker acid, 5 per cent sulfuric acid, we obtain similar results as to the yield of yeast, but in this case we obtain (Section B, Table IV) a yeast of the high nitrogen content in the case where the medium is highest in both total and amino nitrogen. The results in Section B, Table IV, may perhaps in part be ascribed to the fact that the total amount of nitrogen was not the same in each case. However, it is not likely that the variations indicated, entirely account for the results of yeast growth. It is more likely that the stage of hydrolysis is the more important factor. In fact the conclusions arrived at in regard to the action of asparagine and aspartic acid might possibly be applied here, that is, both amino and amide nitrogen are desirable in a medium for yeast growth. By varying the concentration of the 148 hour hydrolyzed edestin (Section C, Table IV) we obtain results which again show that although the concentration of the completely hydrolyzed protein is an important factor in the yield of yeast, still it never is as efficient as the partially hydrolyzed protein no matter whether in higher or lower concentration. The growth test with 2.1 mg. of nitrogen in the form of completely hydrolyzed edestin (Section C, Table IV) shows that it increases the yield of yeast over the control test 2.76 times whereas the relative concentrations in nitrogen are only as 1 to 1.76. It shows the importance of the two forms of nitrogen, that is, in the vitamine solution and the hydrolyzed edestin, respectively.

Alkaline hydrolysis (Section D, Table IV) of edestin yields a poor nutrient both from the standpoint of yield and nitrogen content of the yeast. However, here we again observe that the shortest period of hydrolysis gives the most efficient medium and this in spite of the fact that the amino nitrogen content was very low and practically the same in each case. The comparative tests tabulated in Section E, Table IV, confirm the previous results, but also show that an alkali-hydrolyzed edestin may be converted into a better yeast nutrient by a subsequent hydrolysis by sulfuric acid; both the yield and the nitrogen content of the yeast were increased very decidedly. Whether the result is due to increased amino nitrogen or to changes in optical activity or both must be determined by other studies.

The studies with hydrolyzed edestin show that a partial hydroly-

sis is desirable, but that a complete one is very undesirable. Possibly this is due to the greater availability of certain amides or peptide linkings for yeast growth, but it may also be due to the partial or complete destruction of certain amino-acids or to the loss of some of them through precipitation in the process of removal of  $\text{SO}_4$  and Ba ions. Tryptophane would be completely destroyed in the acid hydrolysis, while cystine and tyrosine would tend to be precipitated in the removal of  $\text{SO}_4$  and Ba ions. There are, of course, other unknown groups in the edestin molecule which may also be destroyed in the process of hydrolysis.

*The Action of Certain Amino-Acids on Yeast Growth.*—The results of these studies are given in Table V. If we compare the yield of yeast in the control test, in the absence of hydrolyzed edestin, with the action of the pure amino-acids in the presence of the vitamine solution in both cases, we find that in the low concentration employed cystine, histidine, glucosamine, and cystine in the presence of tyrosine each retard the growth of yeast, the yield ranging from 37 to 96 per cent of the control test. With cystine alone the effect on the nitrogen content of the yeast is very marked also. In every case, except cystine alone, the higher of the two concentrations of amino-acid employed had the more inhibiting effect. The most marked retarding effect was shown by the mixture of cystine and tyrosine, the yield of yeast being decreased in one case to 37 per cent of the control test. Of the other amino-acids used, tyrosine, tryptophane, lysine, and arginine slightly increase the yield of yeast in the absence of hydrolyzed edestin. Here also it may be noted that the higher concentration employed never had the distinctly better effect, in fact in the case of tyrosine the higher concentration almost retarded growth. Proline in the concentrations employed was without action. Apparently none of the amino-acids studied, except tyrosine in low concentration, has a distinctly beneficial effect when used together with the vitamine in an otherwise synthetic medium. The retarding action is much more marked. Whether this toxic action is due to the amino-acid itself, to the deaminized product, or to the corresponding alcohol as suggested by some workers is not determined here.

The effect of the amino-acids in the presence of hydrolyzed edestin is a very different story. Here we find cystine a marked

stimulant which increased with the higher concentration employed. Tyrosine and cystine acting together had a similar effect, but to a less degree. Tyrosine and proline separately, stimulated distinctly better in the lower than in the higher concentrations. The same is true to a very much less degree for lysine and arginine. Histidine in the lower concentration had no effect, but in the higher concentration it distinctly lowered the yield of yeast. Tryptophane stimulated slightly in the concentrations employed. Glucosamine was practically without effect. One very interesting finding is that in general there is a tendency that the percentage of nitrogen in the yeast grown in presence of the amino-acid and edestin is higher than in the control test. This is especially true with cystine, tyrosine, histidine, lysine, and arginine. It is not so well shown with tryptophane and proline. These results with pure amino-acids confirm the results obtained with the variously hydrolyzed edestin preparations and certainly show very clearly how very easily an otherwise satisfactory medium may be influenced to stimulate or retard yeast growth by the addition of such small amounts as 0.50 to 20 mg. of amino-acid to a 125 cc. volume. Whether the acids themselves or intermediate products in their metabolism are involved in these actions remains to be determined. However, it is very certain that the additional nitrogen found as yeast protein by no means all comes from the added amino-acid in those cases where the distinct stimulation is observed. For instance, where 20 mg. of cystine or 2.33 mg. of cystine nitrogen were introduced we found 3.59 mg. more yeast protein nitrogen; so also where 1.5 mg. of tyrosine nitrogen were added, we had 2.84 mg. additional yeast protein nitrogen formed. In some cases at least the beneficial effect of the amino-acid must be in some other way than as a nitrogen source pure and simple.

#### SUMMARY.

1. In contrast to previous work on nitrogen nutrition of yeast the effect of the yeast growth-promoting vitamine has been controlled in these studies.

2. Yeast grows better in an asparagine-containing medium in presence of ammonium sulfate than in the absence thereof. Similar results are obtained with succinamide, succinimide, and aspartic acid.

3. In an asparagine-containing medium, practically free from ammonia, the yeast obtains its nitrogen mainly from the  $\alpha$ -amino group and only slightly affects the amide group of the asparagine.

4. Continued acid hydrolysis decreases the value of edestin as a yeast nutrient, but mild hydrolysis improves it up to a certain degree.

5. Alkaline hydrolysis of edestin is more destructive than the acid hydrolysis. Subsequent acid hydrolysis improves the edestin as a yeast nutrient.

6. The lowered nutrient value of edestin with more or less complete hydrolysis may be due to many factors. Some of these factors are the loss of certain important amino-acids, the possible importance of certain peptides, the loss of unknown building stones, and the toxic effects of certain free amino-acids or some of their products of metabolism.

7. In the absence of hydrolyzed edestin cystine, histidine, glucosamine, and cystine in the presence of tyrosine, retard the growth of yeast. This may not be true for lower or higher concentrations than those studied here.

8. In the absence of hydrolyzed edestin tyrosine, tryptophane, lysine, and arginine slightly stimulate yeast growth. Higher concentrations of these tend to retard the growth. Proline had no effect.

9. In the presence of hydrolyzed edestin, cystine, tyrosine, or cystine with tyrosine stimulated yeast growth. Tryptophane, proline, lysine, and arginine acted similarly, but to only a slight degree. Slight changes, increase or decrease, in concentration may easily cause a retardation, but the general tendency is toward a retardation in growth with increased concentration of the amino-acid.

10. In those cases where the most marked growth stimulation was observed the amount of additional yeast nitrogen found was considerably more than the nitrogen introduced as amino-acid. This is especially true for cystine and tyrosine.

In conclusion I wish to acknowledge my indebtedness to Professor F. C. Koch for valuable advice given me throughout the research, and to Dr. R. E. Lee of The Fleischmann Co. whose interest in yeast nutrition made this study possible.

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## CHEMICAL STUDIES ON INTESTINAL INTOXICATION.

### I. THE PRESENCE AND SIGNIFICANCE OF HISTAMINE IN AN OBSTRUCTED BOWEL.

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That the systemic symptoms and death following an acute intestinal obstruction are due to the absorption of toxins from the obstructed part of the bowel has been established by the consistent results of all recent workers in this field.

The closed loop operation introduced by Whipple and his associates (1, 2) has shown that these toxins may be developed independently of foods or the special secretion of any one part of the enteric canal; so that they must arise from the mucosa and its secretions or the bacteria in the lumen of the gut.

The bacteria of the normal gut multiply greatly in the closed gut (3) and the proteolytic strains completely overgrow all others (4); decarboxylating organisms, mainly of the *coli* group, which produce histamine under conditions that prevail in a closed loop, have been isolated from feces and intestinal contents (2, 5, 6, 7); and histamine has been found in normal feces (4, 8) and mucosa (9). True exotoxins are not present in loop fluid (10, 11), so that the amine (or proteose) nature of the toxic agents is strongly indicated.

The loop toxin is absolutely identical with histamine in its pharmacologic action (12, 13, 14), except that it renders blood incoagulable; and, also like it, resists the action of heat (15, 16, 17, 18), acid (refluxing with 20 per cent HCl)<sup>1</sup> alkali, trypsin, and erepsin (2, 19, 20, 21). When loop fluid is treated with alcohol or

<sup>1</sup> Personal communication from Dr. L. R. Dragstedt confirmed by the author. He found the minimum lethal dose for guinea pigs to increase from 3 to 5 cc. after the acid treatment.

half saturated with ammonium sulfate, part of the toxicity appears in the precipitate, as would a proteose, and part remains in the filtrate, as would an amine.

Nesbitt (22) found choline and neurine in obstruction fluid after feeding yolk. Kukula (15) isolated a pentamethylene diamine (cadaverine?) from fluid above an obstruction. Roger (23) showed a proteose present in similar material, and Whipple later secured evidence of a primary heteroproteose (24). Sweet, Peet, and Hendrix (25) found in obstruction fluid a proteose and a "choline-like" substance. Taylor and Pearce (26) were unable to demonstrate histamine in loop fluid by Kutscher's tannic acid method, possibly because the amine was carried down in the tannic acid precipitate—as in solutions of low acidity.<sup>2</sup>

In view of its importance, analysis of obstruction fluid for histamine by more adequate methods was undertaken in this investigation.

### *Methods.*

Histamine was tested for in loop fluids by chemical analysis, by biological assay on strips of intestine, and by intravenous injection in dogs. For chemical analysis the quantitative method of Koessler and Hanke (27, 28, 29, 30) and Koch was used. This consists, briefly, of acid hydrolysis of the protein material, distillation of ammonia, precipitation of the amines and amino-acids with acid phosphotungstic acid, decomposition of this precipitate with baryta, concentration, addition of NaOH to the concentrate, and repeated extraction of the alkaline solution with amyl alcohol to separate the histamine from histidine. Any interfering substances still present are removed by treatment with AgNO<sub>3</sub> and baryta. The histamine is tested for colorimetrically by coupling with diazobenzene sulfonic acid. As little as 0.001 mg. of histamine dihydrochloride (in 8 cc. of test mixture) may be determined quantitatively in this manner.

Guggenheim and Löffler (31) showed the intestine of the guinea pig affords a very satisfactory test for histamine. Longitudinal pieces suspended in warm oxygenated Ringer's solution respond to

<sup>2</sup> Meakins and Harington (Meakins, J., and Harington, C. R., *J. Pharmacol. and Exp. Therap.*, 1922, xviii, 455) have just reported finding histamine in human obstructed colons.

concentrations of histamine as low as 1 part to 250 million. In using this test, the test solutions were carefully neutralized and any preservatives removed, as these factors modify the contraction of the gut.

Injections in the dog were made directly into the femoral vein. Blood pressure was taken from the carotid artery.

#### EXPERIMENTAL.

##### *Histamine Present in Fluid Accumulated in Closed Washed Loops of Jejunum.*

*Sample 1.*—A closed washed loop of the jejunum was made and continuity of the gut reestablished by end to end anastomosis. The dog ran the usual course and died in a typical manner. 90 cc. of chocolate brown fluid with a putrid odor were collected at autopsy and at once boiled. 6 gm. of dark brown solid were obtained by evaporation. 1 gm. of the powdered solid was refluxed (just kept simmering) for 100 hours with 20 per cent HCl. Considerable carbonization occurred despite careful heating, and a large amount of gummy melanoid material which was refractory to the further treatment was formed. The hydrolysate was distilled twice *in vacuo*, filtered from the melanoid matter, slightly acidified with HCl, evaporated, and precipitated with phosphotungstic acid, etc., as indicated above. By double extraction with amyl alcohol both a histidine fraction and a histamine fraction (Solution 1C) were obtained, which were diluted, transferred to precision cylinders, and tested. 1 cc. of Solution 1C gave a color value of 1.1 mm., corrected, which equals 0.0014 mg. of histamine dihydrochloride (by table). The number of mg. of histamine dihydrochloride per 100 cc. of original fluid equals  $0.0014 \times 25 \times \frac{15.2}{7} \times$

$$\frac{10}{9.1} \times \frac{100}{15} \text{ equals }^3 0.6 \text{ mg.}$$

Solution 1C was further tested for histamine by its action on guinea pig gut. 4 cc. were added to the 50 cc. of oxygenated Ringer's solution in which the intestine was suspended. The concentration of histamine in this solution, as given by the colorimetric test, is 1:10,000,000, and the contraction of the gut following its addition was similar to that following the addition of known histamine in like quantity.

Solution 1 C also caused a fall in the blood pressure of a dog when injected intravenously. The shape of the curve was identical with one produced by the injection of known histamine solutions.

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<sup>3</sup> Total volume of Solution 1 C equals 25 cc.  $\frac{15.2}{7} \times \frac{10}{9.1}$  represents dilutions in process of analysis, 1 gm. of solid was equivalent to 15 cc. of the original fluid.

*Histamine Removed by Melanin.*

*Sample 3.*—10 mg. of histamine dihydrochloride were added to 1 gm. of Solid 1, and the whole analyzed parallel with it as a check upon it. Carbonization and melanin formation occurred during acid hydrolysis just as with Sample 1. Only about half (4.4 mg.) of the histamine dihydrochloride added was recovered in the final fraction.

The method used had proved quantitative when tested on pure imidazole solutions, histamine is not easily destroyed even by vigorous chemical treatment (28), and Hanke and Koessler (30) report that histamine is adsorbed by charcoal. In this sample the charred melanoid material formed during hydrolysis removed the histamine by adsorption. (Further evidence for this is afforded by Sample 5.) In Sample 1 also, a considerable quantity of histamine was probably removed in a like manner.

*Alkaline Hydrolysis of Loop Fluid Does not Permit a Quantitative Determination of Histamine.*

*Sample 6.*—10 cc. of fresh jejunal loop fluid were allowed to stand with 3 gm. of NaOH, and the mixture was extracted with amyl alcohol and sulfuric acid, neutralized, dried, and tested, as above. A similar portion with 1 mg. of histamine dihydrochloride added was treated in like manner. In both cases a large amount of brown material was formed and the amyl alcohol extracts were deeply colored (reddish brown). The color did not pass into the acid. Both samples gave tests for histamine but not quantitatively.

*Presence of Histamine in Loop Fluid Confirmed by Further Purification.*

*Sample 2.*—A closed washed loop of the ileum was made and continuity of the intestine reestablished as before. At autopsy 130 cc. of reddish brown fluid were collected and at once evaporated to dryness. 7 gm. of dark brown solid were formed. 2 gm. of this were subjected to acid hydrolysis, etc., exactly as was Sample 1. The histamine fraction was tested colorimetrically.

0.5 cc. gave a color test of 4 mm., corrected, which equals 0.55 mg. histamine dihydrochloride for the sample taken.

1.0 cc. gave a color test of 6 mm., corrected, which equals 0.40 mg. histamine dihydrochloride for the sample taken.

The average of these figures taken showed 1.3 mg. of histamine dihydrochloride per 100 cc. of original fluid.

As pointed out by Koessler and Hanke (27), the failure of twice the quantity to give twice the color value indicated interference with the coupling reaction by some substance. A further purification with  $\text{AgNO}_3$  and baryta was resorted to, and histamine again demonstrated in the silver-insoluble portion by all tests, as in Sample 1.

In the above analysis all the discarded fractions were tested for toxic material by intravenous injection into a dog. None of these fractions caused a fall in blood pressure.

*Histamine in Loop Fluid Determined Quantitatively.*

The above analyses show only the minimum quantity of histamine, for much is removed by the melanin formed in hydrolysis. To avoid formation of this the undried fluid was used for hydrolysis. The following group of analyses done in this way yielded quantitative results.

*Sample 7.*—200 cc. of chocolate brown fluid were obtained at autopsy from a closed jejunal loop of 3 days standing. This was centrifuged to remove solid particles and the opalescent supernatant liquid used for this test. 10 cc. were at once refluxed with 37 per cent  $\text{HCl}$  for 40 hours (Sample 7A). No gummy material formed. A second 10 cc. portion was similarly treated after the addition of 10 mg. of histamine dihydrochloride (Sample 7B); and the hydrolysis again accomplished without carbonization. The subsequent treatment paralleled that of Sample 1. 1 cc. of the final histamine fraction for Sample 7A gave a color test of 15.7 mm., corrected, which by table corresponds to 0.021 mg. of histamine dihydrochloride, or 0.3 mg. of histamine dihydrochloride for the original 10 cc. sample. The final fraction of Sample 7B was so concentrated that 0.05 cc. gave a color test of 23.5 mm., corrected, which equals 0.0313 mg. histamine dihydrochloride, or 9.8 mg. for the whole sample taken. 0.3 mg. originally present in the fluid is included here, so 9.5 mg. of the added histamine dihydrochloride were recovered. This is 95 per cent of the total; and is within experimental error for a concentrated solution.

The solid thrown down by centrifugalization was roughly analyzed for histamine to eliminate any loss by occlusion. The solid was suspended in 25 cc. of water and 10 cc. used for extraction with amyl alcohol (3 portions of 20 cc. each) and this in turn with  $\text{N H}_2\text{SO}_4$  (3 portions of 10 cc. each). The combined acid extracts were treated as usual. 1 cc. of the histamine fraction had a color value of 4.1 mm., corrected, which equals 0.0055 mg. of histamine dihydrochloride, or 0.2 mg. for the whole sample of loop fluid. There was present in the 200 cc. of fluid, therefore, at least the equivalent of 6.2 mg. of histamine dihydrochloride.

These various histamine fractions gave the typical depressor effect when injected into dogs.

*Sample 9.*—A closed loop of the upper jejunum was made by the usual operation. 44 hours later the dog was very ill and could not stand. It was sacrificed, and 40 cc. of bloody brown foul smelling fluid were obtained from the distended loop. 15 cc. of this were at once refluxed for 40 hours with 37 per cent HCl, and the further procedure of Sample 1 was followed. The histamine fraction finally secured was tested in the usual way. 1 cc. gave a color value of 18.5 mm., corrected, which equals 0.025 mg. of histamine dihydrochloride, or 0.3 mg. for the sample taken. This would correspond to 0.8 mg. for the original fluid obtained. This fraction also gave a typical histamine depression of blood pressure.

*Sample 10.*—A closed loop of the upper jejunum was made as usual. The dog became progressively weaker and died in 4 days. 125 cc. of bloody brown foul smelling fluid were obtained at autopsy. 15 cc. were at once refluxed with acid and carried through the usual procedure. The histamine fraction finally obtained was tested colorimetrically. 1 cc. gave a color value of 23 mm., corrected, which equals 0.031 mg. of histamine dihydrochloride, or 0.4 mg. for the sample taken. The total fluid obtained contained 3.3 mg. of histamine dihydrochloride.

In the above analyses the time of appearance and shade of the color were correct for histamine.

#### *Histamine Present in Material Accumulated in Closed Washed Loop of Colon.*

Acute obstruction in the lower bowel, especially the colon, is not attended by such a fulminating syndrome as is higher obstruction. Many human cases of obstipation lasting for months are known; and closed loops of the colon in dogs have been found by many workers (32, 33, 34) to be entirely compatible with life. In such loops of colon, however, a fecal-like mass accumulates which is extremely toxic and, upon intravenous injection in dogs, shows a typical depressant action. The following analysis shows the presence of histamine in such material.

*Sample 8.*—A closed loop of the colon was made in the usual manner.<sup>4</sup> The dog made an uneventful recovery and showed no symptoms for 8 months, when it was again operated upon and the closed loop removed. It contained 100 gm. of a grey buttery solid. This was triturated with water and the suspension found to give a typical loop toxin fall in blood pressure upon injection. 10 cc. of the suspension (equals 1 gm. of solid) were refluxed with 37 per cent HCl for 45 hours. Subsequent treatment as above yielded a histamine fraction, 1 cc. of which gave a color value of 7.5 mm., corrected, which equals 0.01 mg. of histamine dihydrochloride, or 0.14 mg. for the sample taken. There was present in the original sample, therefore, at least 14 mg. of histamine dihydrochloride.

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<sup>4</sup> Operation performed by Dr. L. R. Dragstedt.

*Histamine Present in Sterile Jejunal Mucosa but Not in Sterile Jejunal Secretion.*

The aseptic formation of histamine in the body has not been conclusively demonstrated. Barger and Dale (9) found this amine in washed ox mucosa which had, however, been harboring proteolytic bacteria. The evidence presented by Abel and Kubota (35) favoring the formation of histamine during normal metabolic processes, has been questioned by Hanke and Koessler (36).

Much evidence indicates that the obstruction toxins are in part, at least, the result of bacterial activity and formed only in their presence (18, 33, 37, 38, etc.). The following experiment affords further evidence on these points.

*Sample 11.*—The usual operation for a closed loop of the jejunum was performed, and an end to end anastomosis made. The isolated segment of gut attached only by its mesentery, was not closed by infolding the ends however, but left open. Further exposure of the mucous surface (to the sterilizing action of the peritoneal secretions, 33) was secured by a slit along the antimesenteric border of the loop, which allowed it to evert. The mucosa was well washed with sterile water, rinsed with ether, and dropped back into the abdomen, which was then closed. The dog made a perfect recovery and remained normal. 149 days later she was still entirely normal (3 days before being sacrificed she was observed in coitus), and was sacrificed. The autopsy was performed immediately under aseptic conditions. The peritoneum and mesenteries were moderately congested and were besprinkled with fibrin-like granulations, though no adhesions between them were found. The open loop was adherent about its edges but the mucosa was freely exposed in the cavity. The mucosa appeared entirely normal on gross and microscopic examination. Within the peritoneal cavity were about 200 cc. of light grey mucilaginous material, the accumulated secretion of the everted mucosa. Cultures of the mucosa and secretion taken on agar, blood agar, and Endo plates, agar slants, and in broth were all negative. The whole abdomen presented a strikingly pathological picture, yet the dog was in good health when sacrificed.

10 gm. of the mucous secretion were at once refluxed with 40 cc. of 20 per cent HCl for 33 hours, and the usual analysis was carried out. 1 cc. of the final histamine fraction gave a color value of about 15 mm. but *the color was distinctly green*. Intravenous injection into a dog of the original material, or of the histamine fraction, had no effect on blood pressure. Histamine was therefore absent from the sterile jejunal secretion.

The mucosa of this sterile loop was thoroughly washed, scraped off, minced, and at once refluxed with 25 cc. of 20 per cent HCl for 33 hours. Its moist weight was 6 gm. The usual analysis was performed and the final histamine fraction secured. 0.25 cc. of this solution gave a color



value of 14 mm., corrected, which equals 0.019 mg. of histamine dihydrochloride, or 10 mg. for the sample taken. On intravenous injection into a dog this solution gave a typical histamine depression similar to one produced by known histamine of like strength.

The sterile mucosa of the dog's jejunum, therefore, may contain histamine. No explanation is offered. The experiment is being repeated to determine the constancy of this condition.

*Histamine Present in the Mucosa of a Closed Jejunal Loop.*

The rôle of the intestinal mucosa in the formation of loop toxin has been especially emphasized by Whipple, who believes the toxin to be a perverted secretion (21). The presence of histamine in loop mucosa as shown below is of interest in this connection, and as a check on the above.

*Sample 10.*—The mucosa of Dog 10 in the above series was used. At autopsy the closed loop was found distended but not perforated. The mucosa was somewhat injected, but otherwise normal. The ends which had been infolded and tied were gangrenous. The end to end anastomosis showed a small perforation, and a hemorrhagic peritonitis had developed.

The mucosa was carefully washed free of all contents and partly dried. 6 gm. were refluxed with 37 per cent HCl, and carried through the remaining procedure as above. The final histamine fraction was tested as usual. 1 cc. gave a color value of 15 mm., corrected, which equals 0.02 mg. of histamine dihydrochloride, or 0.3 mg. for the whole sample.

*Histidine Present in Loop Fluid and Mucosa.*

The histidine content of some of these samples was determined in the manner indicated under the analysis of Sample 1. Following are the results.

Sample 2 contained 78 mg. of histidine dihydrochloride per 100 cc. of fluid, or 101 mg. for the entire quantity.

Sample 8 contained 1.5 mg. of histidine dihydrochloride per gm. of solid, or 150 mg. for the entire quantity.

Sample 9 contained 73 mg. of histidine dihydrochloride in 100 cc. of fluid, or 29 mg. for the entire quantity.

Sample 11 (mucosa) contained 2.4 mg. of histidine dihydrochloride for the entire 6 gm. of moist mucosa.

Sample 11 (fluid) contained 14.1 mg. of histidine dihydrochloride in 10 gm. of jelly, or 280 mg. for the entire quantity.

Sample 10 (mucosa) contained 31.4 mg. of histidine dihydrochloride for the entire 6 gm. of moist mucosa.

Sample 10 (fluid) contained 30 mg. of histidine dihydrochloride in 100 cc. of fluid, or 37 mg. for the entire quantity.

Such widely varying figures obtained under dissimilar conditions are too few to permit any generalizations, though it might be noted that Sample 11 (mucosa) which was rich in histamine is poor in histidine. In all cases, however, there is present an ample supply of histidine to account for the presence of the histamine if the latter were entirely derived from it by decarboxylation. The sequence is thus practically complete; from the presence in closed loops of histidine-rich substrates and histamine-forming bacteria, to the actual isolation of the histamine so formed. Since histamine is present in the normal gut, obstruction must involve an increased production of this amine, a change in its absorption or detoxication by the mucosa, or all these factors; else no systemic symptoms could appear.

Histamine is probably only one of the amines formed by protein decomposition and is more important only in that it is the most active. The other putrefaction products must all contribute their toxicities to the total.

*Combined Histamine also Present in Loop Fluid. Evidence for a "Peptamine Histamine."*

Proteoses as well as amines may play a significant part in the obstruction toxemia. A toxic proteose fraction has been isolated from loop fluid by Whipple and his collaborators (24), which resembles loop toxin in activity, including the anticoagulant action. Also it is alcohol-insoluble, as part of the loop toxin is. Certain properties, however, the two do not appear to possess in common, for no proteose can resist the chemical treatment which the loop toxin withstands, for example boiling with 20 per cent HCl. These facts would all be brought into harmony by the presence of a histamine proteose of the nature of the peptamines synthesized by Guggenheim (39), and which gave a histamine-like action.

Bacteria having the power of decarboxylating free histidine might well attack the exposed carboxyl group on a histidine molecule attached by its imidazole group to a protein chain. A compound, such as would be formed by this process, should be

alcohol-insoluble, non-diffusible through collodion, yield histamine upon hydrolysis, and show a histamine-like pharmacodynamic action with a peptone-like action (*e.g.* anticoagulant) superimposed. Acid hydrolysis would not materially decrease its toxicity, as the histamine so liberated would compensate for the proteose linkings destroyed. These are exactly the properties of one of the loop toxins.

The red dye used as a test for histamine is formed by the coupling of the diazotate (or its tautomeric nitrosamine) with the imidazole nucleus. The peptamine above postulated would have this nucleus combined with the peptide chain, and should give a feebler color test for histamine than would the hydrolysate. This difference between free and combined histamine, and their different alcohol solubilities, make possible the following analysis for the peptamine form.

*Sample 5.*—5 mg. of histamine dihydrochloride were added to 50 cc. of fresh fluid from a closed loop of jejunum, and the whole was boiled. Coagula which formed were removed by centrifuging the whole after 24 hours; washed; and the washings added to the original red opalescent supernatant liquid. Over half the added histamine dihydrochloride was carried down in the coagulum, as only 2.4 mg. were subsequently recovered. This shows that the losses in the early experiments, recorded above, were indeed due to adsorption. The fluid and washings (45 cc.) were treated with 5 volumes of 95 per cent alcohol and the whole was allowed to stand over night. A gummy orange precipitate formed, which was filtered from the alcohol solution and washed. The alcohol filtrate was clear and of a mahogany color. Analysis showed this to contain all the free histamine recovered.

The alcohol precipitate was extracted with boiling water, and the solution divided into 2 parts. One was at once extracted with amyl alcohol and acid, and the final histamine fraction tested colorimetrically. *There was no color formed.* The other part was hydrolyzed by refluxing for 70 hours with 20 per cent HCl and then distilled *in vacuo* twice as with Sample 1. The residue from the second distillation was extracted with amyl alcohol and acid. The histamine fraction finally obtained gave a color test corresponding to 0.6 mg. of histamine dihydrochloride for the alcohol precipitate. This precipitate, therefore, contained no free histamine before hydrolysis but did contain some after hydrolysis.

*Sample 7.*—A portion of this was tested by Koessler and Hanke's method for histamine, as detailed above. 30 cc. of the remainder were treated with 5 volumes of 95 per cent alcohol and allowed to stand over night. A precipitate was removed by filtration, and suspended in water (Fraction 7PS). This caused a slight drop in blood pressure on intravenous injection, but not so marked as that caused by the alcohol filtrate (Fraction 7FA) which contained the free histamine (See Fig. 1).

Part of the suspension (Fraction 7PS) was at once extracted and a histamine fraction secured. This gave a slightly green color test which, if all due to histamine, would correspond to 0.25 mg. for the whole alcohol precipitate. The remainder of the suspension was hydrolyzed by refluxing with 20 per cent HCl for 35 hours, and then treated as the similar fraction in Sample 5. The final histamine fraction gave a color value corresponding to 1.0 mg. of histamine dihydrochloride for the whole alcohol precipitate, or 0.75 mg. more than before hydrolysis. This sample, therefore, also shows the presence of combined histamine.

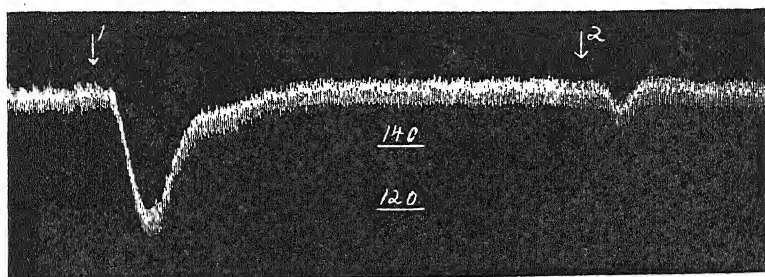


FIG. 1. Typical tracing. Dog. Ether anesthesia. Carotid blood pressure.  $\downarrow^1$  = injection of 1 cc. of the alcohol filtrate of Fluid 7 (Fraction 7FA).  $\downarrow^2$  = injection of 1 cc. of a water solution of the alcohol precipitate of Fluid 7 (Fraction 7PS). 2 cm. = 1 min.

#### SUMMARY OF RESULTS.

Table I shows the presence of histamine in seven out of eight loop fluids analyzed. The fluid that contained none had been formed under sterile conditions and had caused no symptoms. The presence of histamine in sterile as well as loop mucosa, however, forbids the conclusion that this amine can be formed *only* through the agency of bacteria. The presence of a peptamine histamine is shown for two samples of loop fluid. The chemical properties and biological action of closed loop fluids may be accounted for by their content of free and combined histamine, though other toxins surely are present.

#### CONCLUSIONS.

1. Histamine is present in the contents of isolated closed loops of the large or small intestine. The amount varies in different samples; 2 to 3 mg. of histamine dihydrochloride per 100 cc. of fluid being a fair minimum average.

TABLE I.

Sample of loop fluid.	Total volume.	Total content of histidine dihydrochloride.	Content per 100 cc. of histidine dihydrochloride.	Total content of histamine dihydrochloride.	Content per 100 cc. of histamine dihydrochloride.
	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	90			0.5	0.6
2	130	101	98	1.7	1.3
6				Plus.	Plus.
7	200			6.2	3.1
8	100 (gm.)	15	15 (per 10 gm.)	14.0	1.4 (per 10 gm.)
9	40	29	73	0.8	2.0
10	125	37	30	3.3	2.7
11	200	280	140	0.0	0.0
10 Mucosa.	6 (gm.)	31	52 (per 10 gm.)	0.3	0.5 (per 10 gm.)
11 "	6 (gm.)	2.4	4 (per 10 gm.)	10.0	16.7 (per 10 gm.)

Sample of loop fluid.	Total combined histamine in alcohol-insoluble portion. Histamine dihydrochloride.
	<i>mg.</i>
5	0.6 (from 50 cc. of fluid.)
7	0.75 (from 30 cc. of fluid.)

2. A combined histamine derivative is also present in obstruction fluid. Evidence is presented that this is of peptide nature.

3. The sterile secretion of jejunum contains no histamine (1 experiment.).

4. Sterile mucosa of the jejunum contains histamine (1 experiment); as does the mucosa of closed loops of the jejunum (1 experiment).

5. Histidine is present in loop fluid and mucosa.

6. Histamine is carried down in heat and acid coagula of loop fluid, and small amounts may be adsorbed in the alcohol precipitate from loop fluid.

The writer wishes to express his indebtedness to Professors A. J. Carlson and L. R. Dragstedt for much valuable aid and counsel throughout this work, to Professor F. C. Koch for pure histidine dihydrochloride kindly supplied by him and for other kindnesses, and to Drs. M. T. Hanke and K. K. Koessler for

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# THE UTILIZATION OF CALCIUM AND PHOSPHORUS OF VEGETABLES BY MAN.

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The mineral metabolism in diabetes has assumed greater importance because of the recent tendency to prescribe a lower intake of protein, as suggested by Newburgh and Marsh (1920). This lowering of the protein entails a consequent reduction of the phosphorus. Typical diabetic diets in this Clinic show protein intakes of from 25 to 60 gm. daily. It has long been customary to allow diabetics large amounts of vegetables because of their low content of carbohydrate. 400 to 800 gm. are the ordinary amounts allowed. With such diets the question arises: Are the calcium and phosphorus available for the needs of man? We know from the experiments of Rose (1920) that the calcium of carrots is well utilized by man, as contrasted with the results obtained by McClugage and Mendel (1918) who found poor utilization of the calcium of carrots and spinach when fed to dogs. The present authors have been unable to find any experiments dealing with the availability of vegetable phosphorus for man. It is perhaps not generally known that the phosphorus of washed wheat bran, which is often fed to diabetics, is not available; whereas a certain part of the phosphorus of unwashed bran is assimilated.<sup>1</sup> It occurred to us that vegetables containing much cellulose might also give a similar result when eaten by man. In view of the foregoing points, investigation of this problem seemed desirable.

## *Plan of Experiment.*

Two healthy, young women volunteered as subjects for the study. Subject O was 24 years of age and weighed 57 kilos,

<sup>1</sup> Unpublished observations from this laboratory.



while Subject L was 29 years old and weighed 57.3 kilos. These subjects engaged in their usual activities, one as a nurse and the other as a laboratory assistant.

The diet in the first period of 4 days was so planned as to approximate as closely as possible the maintenance requirements for calcium, phosphorus, and nitrogen as given by Sherman (1920, *a*, *b*, and *c*). For the second period of 7 days, the diet of Subject O was altered by the substitution of 0.5 gm. of phosphorus with an equivalent amount of phosphorus from vegetables. The diet of Subject L in the second period differed from that of the first by the addition of 0.5 gm. of vegetable phosphorus.

### *Methods.*

The following analytical methods were used in this study: calcium, McCrudden (1909-10 and 1911-12); phosphorus in urine, Bell and Doisy (1920); phosphorus in feces, Meigs, Blatherwick, and Cary (1919); nitrogen, Folin and Wright (1919); and pH, Palmer, Salvesen, and Jackson (1920-21).

The commonly accepted values, as given in the different books by Sherman, were used in computing the calcium and phosphorus contents of the diets. Separation of the feces was accomplished with the aid of charcoal.

Of the vegetables eaten: lettuce was taken uncooked; asparagus was of the canned variety; celery and spinach were cooked, and the excess fluid concentrated so that none was lost; summer squash was steamed; and the cabbage was boiled. The juice from the latter was drunk by Subject L but was refused by Subject O. Tests made upon this juice revealed only a trace of phosphorus by the colorimetric method. Distilled water was used in cooking the vegetables, and only distilled water was drunk. Chemically pure sodium chloride was also used throughout.

Table I shows the kinds and amounts of foods consumed in the two experimental periods.

### *Results.*

In Tables II and III are given the daily values obtained for the urines of the two periods. It will be noted that the pH of the urines resulting from the vegetable period are distinctly less acid, as would be expected from the preponderance of base-forming

TABLE I.

*Kinds and Amounts of Foods Eaten, with Daily Intake of Calories, Protein, Calcium, and Phosphorus.*

	Subject O.		Subject L.	
	Period I.	Period II.	Period I.	Period II.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Bread.....	100	150	150	150
Beef, lean.....	100		100	100
Milk.....	150		220	220
Egg yolk.....	25			
Butter.....	105	105	105	105
Sugar.....	40	40	20	30
Apple, edible portion.....	160	160	160	160
Potato, edible portion.....	100	50	100	100
Cream, 36 per cent.....	30	30		
Coffee.....				
Tea.....				
Egg white.....		150		
Olive oil.....		20		30
Vinegar.....		25		25
Cabbage.....		200		200
Celery.....		200		200
Squash, summer.....		200		200
Spinach.....		100		100
Lettuce.....		100		100
Asparagus.....		200		200
Calories.....	2,009	2,055	1,777	2,271
Protein.....	48.7	46.0	46.1	56.1
Nitrogen.....	7.79	7.36	7.38	8.98
Phosphorus.....	0.756	0.751	0.670	1.174
Calcium.....	0.335	0.508	0.358	0.742

elements in these diets. The urine of both subjects on the last experimental day showed lower values for all constituents than on the preceding days. These have not been included in the totals for the period. In computing the totals for Period II, the averages for the first 6 days have been called the elimination for the 7th day and calculations for the period based on these figures. Inclusion of the values as determined would favorably influence all the balances.

Table IV shows the intake, output, and balance of calcium, phosphorus, and nitrogen for the various periods; also the daily

TABLE II.

*Daily Output of Calcium, Phosphorus, and Nitrogen in Urine (Subject O).*

Period.	Day.	Volume.	pH	Ca	P	N
	1921	cc.		gm.	gm.	gm.
I	Dec. 7	700	6.0	0.086	0.53	7.97
	" 8	525	6.6	0.104	0.47	7.82
	" 9	510	5.9	0.103	0.46	8.16
	" 10	1,220	6.0	0.089	0.50	8.25
II	" 11	1,120	6.7	0.116	0.42	8.56
	" 12	1,330	6.9	0.084	0.51	8.54
	" 13	1,095	6.5	0.076	0.37	8.12
	" 14	1,470	6.5	0.099	0.46	8.58
	" 15	1,590	6.6	0.086	0.40	7.71
	" 16	1,170	6.5	0.075	0.51	7.21
	" 17*	1,270	6.4	0.076	0.29	6.05

\* Not used in computing totals. Average of 6 preceding days used for 7th day in calculating totals for period.

TABLE III.

*Daily Output of Calcium, Phosphorus, and Nitrogen in Urine (Subject L).*

Period.	Day.	Volume.	pH	Ca	P	N
	1921	cc.		gm.	gm.	gm.
I	Dec. 7	935	6.1	0.114	0.57	7.61
	" 8	885	5.5	0.098	0.52	6.79
	" 9	560	5.4	0.103	0.41	5.89
	" 10	1,500	6.2	0.155	0.53	6.51
II	" 11	795	6.7	0.108	0.57	6.82
	" 12	1,425	6.6	0.131	0.57	6.61
	" 13	1,690	6.7	0.120	0.55	6.11
	" 14	2,095	6.5	0.139	0.57	6.89
	" 15	1,265	6.3	0.125	0.55	6.08
	" 16	1,710	6.9	0.120	0.55	6.70
	" 17*	1,320	6.5	0.099	0.45	5.68

\* Not used in computing totals. Averages of 6 preceding days used for 7th day in calculating totals for period.

balance of the three elements. Subject O in Period I is seen to have been in decided negative balance. In the following period, in which 0.5 gm. of phosphorus was substituted with an equivalent

amount from vegetables, the calcium and phosphorus balances became positive, while the nitrogen balance remained negative. In the case of Subject L, her phosphorus and nitrogen balances in Period I were slightly negative, and the calcium balance was

TABLE IV.

*Intake, Output, and Balance of Calcium, Phosphorus, and Nitrogen.*

	Subject O.		Subject L.	
	Period I. Dec. 7-10, 1921.	Period II. Dec. 11-17, 1921.	Period I. Dec. 7-10, 1921.	Period II. Dec. 11-17, 1921.
Calcium.				
Food, gm.....	1.340	3.556	1.532	5.194
Urine, gm.....	0.382	0.625	0.470	0.868
Feces, gm.....	2.017	2.396	1.570	2.963
Balance, gm.....	- 1.059	+ 0.535	- 0.508	+ 1.363
Daily balance, gm.	- 0.265	+ 0.076	- 0.127	+ 0.195
Phosphorus.				
Food, gm.....	3.02	5.26	2.68	8.22
Urine, gm.....	1.96	3.12	2.03	3.92
Feces, gm.....	1.53	1.45	0.93	1.51
Balance, gm.....	- 0.47	+ 0.69	- 0.28	+ 2.79
Daily balance, gm.	- 0.118	+ 0.099	- 0.07	+ 0.40
Nitrogen.				
Food, gm.....	31.17	51.52	29.50	62.83
Urine, gm.....	32.20	56.84	26.80	45.75
Feces, gm.....	5.29	9.34	3.45	7.69
Balance, gm.....	- 6.32	-14.66	- 0.75	+ 9.39
Daily balance, gm.	- 1.58	- 2.09	- 0.19	+ 1.34
Remarks.	Basal diet.	0.5 gm. P of Diet I substi- tuted with 0.5 gm. veg- etable P.	Basal diet.	0.5 gm. vegetable P added to Diet I.

definitely negative. Upon adding 0.5 gm. of phosphorus from vegetables in the following period, the balances for all three elements became strongly positive. Separation of the feces of Subject O in Period I was not definite, and it is possible that this

accounts for the rather larger negative balances of calcium and phosphorus. However, if computations are made for the whole experiment, Subject O showed equilibrium for phosphorus, a daily negative balance of 0.048 gm. of calcium, and a decidedly negative nitrogen balance. The diet of this subject was probably of insufficient caloric value, as indicated by the loss of nitrogen but even under these conditions she was able to maintain phosphorus equilibrium and to approximate calcium equilibrium when the greater part of those elements was derived from vegetables. It would appear that the daily intake of calcium in Period I of 0.335 gm. for Subject O and of 0.358 gm. for Subject L was insufficient for maintenance. The relatively short length (4 days) of this period, however, does not allow of definite conclusions.

This study corroborates the findings of Rose (1920) regarding the availability of calcium derived from vegetables. The poor utilization by dogs of the calcium of carrots and spinach observed by McClugage and Mendel (1918) may have resulted from the drying of these before feeding. For instance, Hart, Steenbock, and Hoppert (1921) have recently shown that the calcium of green plant tissue was better assimilated than that of the dried product when fed to goats.

Our results demonstrate conclusively the availability of the phosphorus contained in vegetables.

#### SUMMARY.

Data are presented which indicate that calcium and phosphorus derived from vegetables are capable of meeting the maintenance needs of man.

It is a pleasure to acknowledge our hearty thanks to Miss Florence H. Smith, Dietitian, whose careful supervision of the diets made this study possible.

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## A METHOD OF QUANTITATIVE DETERMINATION OF TRYPSIN.

### A MODIFICATION OF GROSS' METHOD.

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Casein is hydrolyzed by trypsin in alkaline, neutral, or faintly acid solutions. The first product of hydrolysis is paracasein; next, caseoses; and finally, amino-acids. Casein and paracasein are precipitated by acetic acid, but its further hydrolytic products are not. The failure to obtain a precipitate with acetic acid marks the stage at which the last trace of paracasein has been hydrolyzed to the caseoses.

Based on this fact, Gross<sup>1</sup> derived a method for a quantitative determination of trypsin. His procedure is as follows: Into each of a series of test-tubes, 10 cc. of an alkaline casein solution, which has been prepared by dissolving 1 gm. of dry casein and 1 gm. of  $\text{Na}_2\text{CO}_3$  in 1,000 cc. of distilled  $\text{H}_2\text{O}$ , are introduced. To each of these tubes, increasing quantities of trypsin solution are added, and the tubes are incubated in a thermostat kept at  $40^\circ\text{C}$ . After 15 minutes of digestion, a few drops of 10 per cent acetic acid are added to each tube. A turbidity will be produced in those tubes in which digestion is incomplete. The concentration is then calculated from the smallest quantity of the trypsin solution which does not produce the turbidity.

During the course of a study in the nature of proteolytic enzymes, where a large number of digestion experiments had to be conducted, we felt this apparently simple method might be still further simplified. The difficulties with Gross' method are: (1) It is very hard to get an end-point of digestion; (2) it requires too

<sup>1</sup> Gross, O., *Arch. exp. Path. u. Pharmacol.*, 1908, lviii, 157.



many test-tubes; and (3) unnecessarily large amounts of the casein and unknown trypsin solutions are required.

Although the original method is of service the following modification might be found useful.

### *Reagents.*

*The Alkaline Casein Solution.*—Dissolve 0.1 gm. of pure casein in 15 cc. of 0.1 N NaOH. Add 400 cc. of distilled H<sub>2</sub>O and titrate 10 cc. of this solution until just colorless to phenolphthalein, using 0.01 N HCl. Add a corresponding amount of the acid to the remainder and make up to 500 cc. (0.2 per cent casein solution).

*Mixture of Sodium Hydroxide and Acetic Acid.*—100 cc. of this solution contain 17.2 cc. of normal soda and 33.7 cc. of normal acetic acid.

*Standard Trypsin Solution.*—Dissolve 0.01 gm. of good commercial trypsin in 10 cc. of H<sub>2</sub>O; filter it off and add toluene.

If 2 cc. of alkaline casein solution are mixed in a test-tube with 1 cc. of sodium hydroxide-acetic acid mixture, a white cloudiness will appear as usual. When it is digested to caseoses, this white precipitate will no longer be produced. The method is, therefore, to compare the time necessary for the unknown to digest to a point where no turbidity is formed when mixed with acetic acid-sodium hydroxide mixture, to the time required for a known standard trypsin to digest the same concentration of the casein under exactly identical conditions. As will be shown later, the concentration of trypsin of the unknown and the standard are inversely proportional to the time required to complete digestion to a point where no turbidity is produced by mixing the casein and sodium hydroxide-acetic acid mixture.

### *Method.*

Transfer 25 cc. of the stock alkaline casein solution to each of two 50 cc. flasks. Warm to 40°C. in the thermostat. To one of these add 1 cc. of the standard trypsin solution, and to the other, 1 cc. of the unknown, noting the time of this addition. Mix well and replace in the thermostat. At intervals of 5 to 10 minutes, 2 cc. of the digesting mixture are pipetted off from each flask, and mixed with 1 cc. of sodium hydroxide-acetic acid mixture in a

test-tube. Note the time at which the digest fails to give any white precipitate. The standard under this condition will usually require about 15 to 20 minutes. If the unknown requires twice as long as the standard, its concentration is  $\frac{1}{2}$  of the concentration of the standard.

## RESULTS.

*Relation of Concentration of Trypsin and Time of Digestion.*

British Drug House trypsin was used to determine the relation between the concentration of the trypsin and the time of digestion (Table I).

TABLE I.

Test-tube No.	Alkaline casein solution.	Trypsin solution (0.1 per cent solution).	H <sub>2</sub> O added.	Final concentration of trypsin.	Time of digestion completion.	Time $\times$ concentration of trypsin.
	cc.	cc.	cc.	per cent	min.	
1	25	0.25	1.75	0.000925	56	0.0518
2	25	0.5	1.5	0.001851	28	0.0514
3	25	1.0	1.0	0.003703	14	0.0518
4	25	1.5	0.5	0.005555	9 $\frac{1}{2}$	0.0527
5	25	2.0	0	0.007407	7	0.0518

To find the concentration of trypsin in times of British Drug House trypsin, it is only necessary to divide 0.0518 by the time required for digestion to a point of no precipitation.

*Relation between Concentration of Casein and Time of Digestion.*

With the concentration of casein as variable, and that of enzyme as constant, the time of the digestion was studied. The result is given in Table II.

TABLE II.

Test-tube No.	Alkaline casein solution (0.2 per cent).	Trypsin solution.	H <sub>2</sub> O added.	Final concentration of casein.	Time of completion.	Time of completion Concentration of casein
	cc.	cc.	cc.	per cent	min.	
1	15	1	15	0.09668	9	93
2	20	1	10	0.12902	12	93
3	25	1	5	0.16128	15	93
4	30	1	0	0.19354	18	93

## CONCLUSIONS.

From Tables I and II the following relationships are clear:

1. The rate of digestion is simply proportional to the amount of trypsin and the time of the digestion is, therefore, inversely proportional to the concentration of the trypsin, that is, the time of concentration of enzyme multiplied by the time of digestion is a constant.

2. The rate of digestion is inversely proportional to the amount of casein and the time of digestion is, therefore, directly proportional to the concentration of the casein, that is, the time of digestion divided by the concentration of casein is constant.

To determine any concentration of enzyme with any concentration of casein, the following formula might be used:

$C_{\text{casein}}$  = concentration of casein.

$P$  = time required.

$K_1$  and  $K_2$  = constants.

Since  $T \times C_{\text{trypsin}} = K_1$

and  $\frac{T}{C_{\text{casein}}} = K_2$

$$T \times C_{\text{trypsin}} + \frac{T}{C_{\text{casein}}} = K_1 + K_2$$

$$C_{\text{trypsin}} + \frac{1}{C_{\text{casein}}} = \frac{K_1 + K_2}{T}$$

$$C_{\text{trypsin}} = \frac{K_1 + K_2}{T} - \frac{1}{C_{\text{casein}}}$$

# THE PARTIAL PRESSURE OF OXYGEN IN THE BLOOD DURING PROGRESSIVELY INDUCED ANOXEMIA.

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(Received for publication, February 23, 1922.)

We have studied the passage of oxygen through the pulmonary epithelium during progressively induced anoxemia.<sup>1</sup> The dog is remarkably resistant to the effects of a lowered oxygen tension in the inspired air. He will maintain both respiration and an adequate circulation when breathing air containing from 3 to 4 per cent of oxygen. Man, on the other hand, becomes unconscious or "faints" when the oxygen is reduced to between 5 and 8 per cent. Data have not been reported regarding the relationship between the oxygen content of the inspired air which produces unconsciousness in man and that which leads to a stopping of respiration. Comparison, however, indicates that the dog is a most favorable animal for the experimental study of the mechanism controlling the passage of oxygen through the pulmonary epithelium.

Two series of experiments were carried out in a study of the problem. Dogs were operated on under chlorotone analgesia that did not suppress or weaken either respiratory or vascular reflexes. A "rebreather" apparatus was connected by a short tracheal tube and the animal was allowed progressively to exhaust the oxygen from the air enclosed in the system. The carbon dioxide was absorbed by potassium hydroxide and completely removed from the inhalant air. Graphic records of the blood pressure and the

<sup>1</sup> Reported before the 34th Annual Meeting of the American Physiological Society, New Haven, December 27, 1921.

respiration were taken as the animal progressively exhausted the oxygen from the air enclosed in the apparatus and samples of air and arterial and venous blood drawn simultaneously at intervals during the course of the experiments. The blood samples were analyzed for their oxygen content and the air for oxygen and carbon dioxide.

In the first series of experiments analyses were made of the inhalant air. The oxygen content of the inhalant air was, of course, greater than that of a corresponding sample of alveolar air. In the second and more extensive series of experiments, alveolar air was drawn through a small sound inserted deep in the bronchial tree, just short of occluding the terminal bronchus. This method gives a mixed sample of alveolar air which must closely approach the average composition of the air in contact with the pulmonary epithelium. The air samples were analyzed by the Haldane method. The blood analyses were made by a modification of the Van Slyke method. In a few tests, arterial blood samples were also equilibrated against the alveolar air.

The systemic effects of anoxemia are described for both man and dog in the rapidly growing literature. Greene and Gilbert (1921, *a* and *b*) have reported the detailed effect of anoxemia in the production of heart inadequacy in man and (1922) on the heart and circulatory system of the dog. Fig. 34 of their 1922 paper presents the typical course of a progressive anoxemia carried to a fatal termination. The blood pressure, like that of a man undergoing the rebreather test, rises sharply towards the crisis and falls rapidly soon after respiration ceases. The respiratory activity sharply increases both in rate and in amplitude as anoxemia progresses. This has been shown by Greene (1922) for man and dog and by Schneider and his colleagues in the reports from the Medical Research Laboratory of the United States Army (1919). These circulatory and respiratory compensations profoundly influence the partial pressure of oxygen in the alveolar spaces and in the blood. Large respiratory and circulatory minute volumes favor the passage of oxygen through the pulmonary epithelium regardless of the theory proposed to explain such passage.

*Methods.*

*Anesthesia.*—Light chloretone anesthesia or analgesia was used throughout the experiments. Chloretone dissolved in oil was given intraperitoneally in amounts of about 0.3 gm. per kilo. The solubility of chloretone in cottonseed oil varies sharply with the temperature. If the solution is heated and the crystals remelted just previous to use, no difficulty is experienced. Dogs vary in their susceptibility to chloretone (apparently because of the varying amount of stored fat), so that it was the practice to give less than the foregoing dose at the first injection and to supplement this later if desired.

*The Rebreather.*—A rebreather with a 6 liter spirometer was used. Including the dead space of a carbon dioxide absorber and connecting tubes, the total capacity was 7 liters. A two-way flutter valve system controlled the passage of the expired air through the carbon dioxide absorber, which was filled with "shell" potassium hydroxide.

A large straight tracheal cannula was inserted in all cases. A four-way connecting tube permitted the introduction of a tracheal sound for the withdrawal of samples of the alveolar air. The movements of the spirometer served to record the respiratory rate and amplitude. The rebreather was always filled with normal air at the beginning of an experiment.

*Blood Pressure and Blood Samples.*—The blood pressure was recorded from the left carotid artery, the record running synchronously with that of the respiration. The right jugular vein and the right femoral artery were isolated before the experiment was made in order that blood samples could be withdrawn at any time without stasis or delay.

*The Alveolar Air Sample.*—Samples of alveolar air were withdrawn through a No. 33 F. catheter inserted as deeply in the lung as possible. The catheter was inserted until the bronchus was completely occluded and then withdrawn 1 cm., or until the passage of air around the catheter became free. Direct measurement showed insertion to about the level of the xiphoid process. Postmortem examination regularly showed the catheter to be deeply inserted in one of the smaller bronchi of the middle or lower lobe of either lung.

Air samples were collected evenly and regularly through eight or ten respirations. Under these conditions a correct sample of mixed alveolar air was obtained. The greatest danger of contamination was from the dead air of the trachea and the larger bronchi. The deep situation of the catheter in the smaller bronchi tends to guard against such contamination. During the latter two-thirds of expiration the tracheobronchial tree is completely washed out with true alveolar air.

*Blood Oxygen Determination.*—Oxygen determinations were made by the method and apparatus of Van Slyke (1918). This method was chosen rather than that of Barcroft (1914) because of the greater facility of analysis and because the construction of the gas pipette permitted the use of varying quantities of blood for analysis. The Barcroft apparatus is of doubtful accuracy when blood of very low oxygen content is to be analyzed, and the use of larger quantities of fluid than those for which the apparatus was designed is not desirable. The Van Slyke analyzer permits the analysis of double quantities of blood when the effects of low oxygen tensions are to be studied. With 4 cc. samples of blood a smaller amount of ammonia solution was used in order not to disturb the volume relations for which the gas pipette was designed. These larger quantities required more time and greater care in analysis in order to secure the extraction of all the oxygen, but the results were in entire agreement with duplicate analyses of 2 cc. samples. No constant difference was observed between the two sets of analyses.<sup>2</sup>

The tables first published by Van Slyke (1918) permit of the calculation of the oxygen content of a given blood sample with a reasonable degree of accuracy, but certain minor errors are present in this calculation. In our series of analyses, the extracted gases were washed with normal sodium hydroxide to absorb traces of carbon dioxide liberated during the analysis. The oxygen actually present was then determined by absorption with Haldane's alkaline pyrogallol solution. Bohr (1909) reported the absorption coefficient of nitrogen in whole blood as being 0.9 volume per cent,

<sup>2</sup> Van Slyke and Stadie (1921) have described the measurement of small amounts of gas under reduced pressure to permit of more accurate volume readings. Our work was completed before the publication of Van Slyke and Stadie and we were not familiar with this procedure.

but found that venous blood actually contained 1.23 volumes per cent. Buckmaster and Gardner (1912) and later Smith, Dawson, and Cohen (1920) have confirmed these findings of a higher nitrogen content in venous blood than could be accounted for by the absorption coefficient. The residual nitrogen in the gas pipette after absorption of the oxygen was then corrected for the nitrogen present in the blood sample, using the data of Bohr as a standard. The excess of inert gas was considered as representing air arising either from leaks in the apparatus or incomplete extraction of all reagents.<sup>3</sup> The amount of oxygen found was corrected for the presence of this air. A similar correction was made for the amount of oxygen carried in physical solution in the blood. This was calculated from the alveolar oxygen tension and the absorption coefficient of 0.022 for whole blood at 38°C. reported by Bohr (1905). The corrected volume of oxygen was reduced to standard temperature and pressure and the content of the blood sample calculated in terms of volumes per cent (Table I). The oxygen capacity of all blood samples was determined directly and calculated by the same method as the oxygen content.

Oxygen analyses calculated according to this method are from 2 to 3 volumes per cent lower than when calculated according to the original method of Van Slyke. There was no significant difference between the figures for the percentage saturation of the blood obtained by either method of calculation, though the method followed gave rather better agreement between duplicates. The average deviation between duplicate analyses throughout the entire series of experiments was 0.40 volume per cent. The error in the determination of the percentage saturation of the arterial samples, therefore, was less than 5 per cent. Van Slyke and Stadie were able to secure slightly better agreement between duplicate analyses by the use of a small bore gas pipette. The differences, however, are entirely insignificant and the present method of analysis is practically identical with that of Van Slyke and Stadie.

*The Equilibration.*—The oxygen capacity of the blood sample was determined after saturation with atmospheric air at 38°C. according to the usual method.

<sup>3</sup> Van Slyke and Stadie found that the nitrogen content of venous blood was 1.36 volumes per cent. They make no correction for air in the apparatus. These differences in technique are entirely incidental and will have no significant effect on the relative accuracy of the results.



Arterial blood samples were equilibrated with the corresponding alveolar air by the method of MacCallum (1917). 4 cc. of blood, 1 cc. of air-free water, and 45 cc. of alveolar air were transferred to a Van Slyke gas pipette used as a tonometer, and shaken for from 20 to 30 minutes at 38°C. The gas in the tonometer was reanalyzed and the total gas exchange determined. The blood sample in the tonometer was then analyzed directly by the usual

TABLE I.

*Analytical Protocols Illustrating the Method of Calculation of Oxygen Content of Arterial Blood (Dog 9).*

	Experiment 31.		Experiment 32.	
	cc.	cc.	cc.	cc.
Blood sample.....	4.0	2.0	4.0	2.0
Total gas liberated: barometer 743 mm.; temperature 20°C.....	0.660	0.345	0.610	0.285
Residual N.....	0.090	0.060	0.120	0.040
N due to residual air in apparatus.....	0.036	0.033	0.066	0.013
O " " " " " " " calcu- lated from N.....	0.010	0.009	0.017	0.003
O liberated from blood sample.....	0.560	0.276	0.473	0.242
O in physical solution in blood.....	0.011	0.006	0.007	0.003
O chemically bound by hemoglobin.....	0.549	0.270	0.466	0.239
O content of blood sample, vol. per cent....	12.19	11.98	10.34	10.60
Average O content of arterial blood, vol. per cent.....	12.09		10.47	
Average O capacity of blood, vol. per cent	12.77		12.77	
Saturation of arterial blood, per cent. ....	94.6		82.9	
Alveolar O tension, mm.....	100.6		64.0	
" CO <sub>2</sub> " mm.....	33.8		24.9	

method. This method of equilibration was not entirely satisfactory, largely because of the difficulty in completely saturating the blood sample in a tonometer of the dimensions used.

*Method of Sampling.*—All experiments began with a preliminary period with the animal breathing normal air. At a recorded signal, the tracheal cannula was connected to the rebreather and a record was taken during the development of the anoxemia. When the desired level of anoxemia was reached, simultaneous

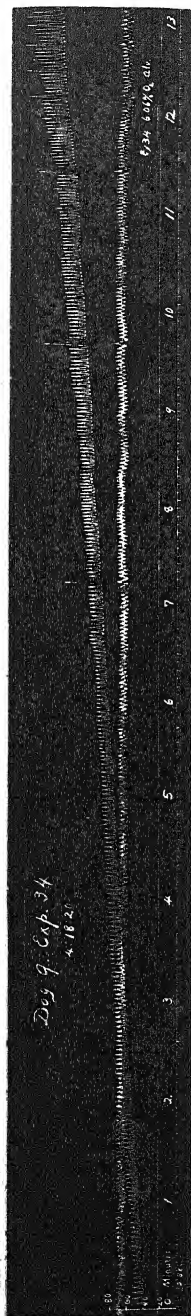


FIG. 1.

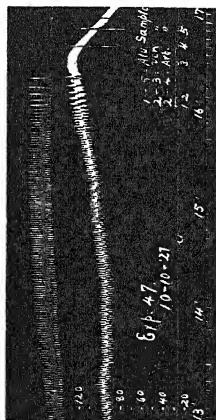


FIG. 2.

FIG. 1. The graphic record of Experiment 34, Dog 9. The statistical data are given in Table III. The alveolar oxygen at the time of drawing blood samples was reduced to 6.06 per cent. This level did not induce circulatory compensations but the respiratory rate was increased slightly and the amplitude greatly as shown in the graph and table. Reduced to  $\frac{1}{4}$  original. Time in 5 second intervals and the minutes are marked.

FIG. 2. This figure represents the terminal portion of the blood pressure and respiratory record and time of drawing samples of Experiment 47. The alveolar air and blood samples are drawn covering the period when respirations stopped. The periods are approximately but not exactly coincident. 1-5 alveolar sample. The drawing began a little earlier and lasted a little longer than the blood samples. The mean should be approximately the same. 2-3 venous blood sample, 2-4 arterial blood sample. These were drawn during the 17th minute as indicated by the signal mark. Respirations reached their maximal rate and amplitude in the 15th minute and stopped at 16 minutes 20 seconds. The maximal blood pressure occurred just following the stopping of respiration as shown. The vagus nerves were cut in this experiment. Time in 5 second intervals.

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TABLE II.

Experiment 33, Nov. 3, 1921. Dog 9. Weight 15.4 kilos. Alveolar oxygen 3.75 per cent, carbon dioxide 2.58 per cent at the time of drawing samples.

Time.	Blood pressure.	Heart rate.	Respiratory rate.	Respiratory amplitude.
				<i>mm.</i>
Normal.	83	99	16	
1 min.	81	108	19	7
4 "	81	108	19	7.6
8 "	82	105	21	8
10 "	83	108	20	9
12 "	84	114	32	11
14 "	85	114	30	16
14 " 30 sec.*	85	112	33	20
16 "	108	78	33 to 16 Slowing.	31

\* Sample drawn during 20 seconds.

samples of alveolar air, arterial blood, and venous blood were drawn. These three samples were taken by separate operators and an attempt was made to draw the samples at as nearly the same time and speed and in as few seconds as was consistent with accuracy. The taking of the three samples was recorded by signals on the

TABLE III.

Experiment 34, Nov. 3, 1921. Dog 9. Weight 15.4 kilos. Alveolar oxygen 6.06 per cent, carbon dioxide 3.08 per cent at the time of drawing samples.

Time.	Blood pressure.	Heart rate.	Respiratory rate.	Respiratory amplitude.
				<i>mm.</i>
Normal.	68	108	22	
1 min.	68	108	24	6.2
4 "	66	108	23	7.4
8 "	74	108	28	9.5
10 "	74	112	28	11.0
12 " *	68	118	27	15.0
13 "	64	111	23	20.0

\* Samples drawn through 20 seconds, blood pressure strikingly uniform, but dropped slightly during drawing sample. The arterial blood was 54.9 per cent saturated (see Fig. 1).

respiratory tracing. After the samples were withdrawn, the tracheal tube was disconnected and the animal allowed to breath normal air while the samples were analyzed and until the time for the test at the level of anoxemia next desired. Analyses were also made during the recovery period following the series of anoxemia tests.

A type record of the entire circulatory and respiratory reactions is given in Fig. 1. The data are assembled in Tables II and III.

#### DISCUSSION OF EXPERIMENTAL PROCEDURE.

Barcroft and his collaborators (1920) have discussed the difficulties incident to the direct determination of the arterial oxygen content as an index to the percentage saturation in the lungs. The chief objection to this procedure arises from the possibility of self-reduction of the blood during the time necessary for analysis. In the present series of experiments the blood was analyzed immediately after withdrawal and in all cases not more than 10 minutes elapsed between the taking of the sample and its analysis. Harrop (1919, *a*) found that mature erythrocytes in man showed no measureable oxygen consumption during 24 hours, and Barcroft presented similar data.

Greater possibilities of error are inherent in the analytical method. All apparatus was calibrated with the greatest care. The gas analyzers gave concordant readings and a single pipette was used for the measurement of the blood samples. Any calibration error in the analyses would be constant throughout the analytical series and should not affect the comparative values obtained. Van Slyke and Stadie (1921) have compared this method of analysis with the Haldane-Barcroft method and report results about 5 per cent higher when using the Van Slyke analyzer. This difference is constant and does not affect the relative values for the percentage saturation of the blood samples.

The percentage saturation of the arterial blood in the normal dog averaged 95.6 per cent in our experiments. This value is on a par with the values found by Hürter (1912), Stadie (1919), and Harrop (1919, *b*), in the blood of normal men.

The character of the respiration has a marked influence on the degree of saturation of the arterial blood. Harrop (1919, *b*) and Barach and Woodwell (1921, *a*) in particular have pointed out the

widely varying arterial saturation associated with alternating periods of apnea and dyspnea. The arterial anoxemia in pneumonia is recognized as owing in large measure to the rapid and shallow respirations. Barach and Woodwell (1921, b) have also described a case of encephalitis with very rapid shallow respirations without pathological changes in the lungs. In this case the percentage saturation of the arterial blood was also greatly reduced.

Doi (1921, b) states that the lowering of the blood pressure following hemorrhage reduced the oxygen intake of the cat. This he brought back to normal by the injection of gum-saline solution

TABLE IV.

*Degree of Saturation of Arterial Blood in Animals Breathing Air Progressively Reduced in Oxygen Tension.*

Dog.	Experiment.	Alveolar air.			Arterial blood.			
		Oxygen.	Oxygen tension.	Carbon dioxide tension.	Oxygen capacity.	Oxygen content.	Saturation determined directly.	Saturation calculated from Barcroft's dissociation curve.
		<i>per cent</i>	<i>mm.</i>	<i>mm.</i>	<i>vol. per cent</i>	<i>vol. per cent</i>	<i>per cent</i>	<i>per cent</i>
9	31	14.38	100.6	33.8	12.77	12.08	94.6	91.0
	32	8.61	64.0	24.9	12.77	10.47	82.9	84.0
	33	3.75	27.9	19.2	12.77	3.66	28.7	30.0
	34	6.06	45.0	22.9	12.77	7.01	54.9	60.0
	36	18.60	138.2	15.5	12.77	12.30	95.0	
11	47	1.90	14.6	14.8	7.88	0.83	10.5	12.0

with the restoration of the original blood pressure. The percentage saturation of the arterial blood changed slightly with the fall in the blood pressure.

In the present series of experiments the relation of the circulatory efficiency to the percentage saturation of the arterial blood is well shown by Dog 9, Experiments 32 (Table IV) and 35 (Table V). The first blood sample was taken at the beginning of a long series of experiments. In the latter experiment the animal was in a weakened condition with the blood pressure running between 50 and 60. Under the strain of the prolonged experimental rebreathing tests, compensation partially failed, and the arterial saturation decreased more rapidly than did the alveolar oxygen tension.

TABLE V.

*Degree of Saturation of Arterial Blood in Animals Breathing Air Progressively Reduced in Oxygen Tension.*

Dog.	Experiment.	Inspired air.		Arterial blood.			Dog.	Experiment.	Alveolar air.		Arterial blood.		
		Oxygen tension.	Oxygen capacity.	Oxygen content.	Saturation.	Oxygen tension.			Carbon dioxide tension.	Oxygen capacity.	Oxygen content.	Saturation.	
		mm.	vol. per cent	vol. per cent	per cent			mm.	mm.	vol. per cent	vol. per cent	per cent	
1	1	159.3	25.1			7	25	85.4	29.5	16.30	10.50	64.5	
	2	21.1	25.1				26	34.7	20.5	16.30	4.25	26.1	
	3	159.3	25.1	24.2	96.4	8	27	133.0	21.1	11.70	11.45	98.7	
	4	22.3	25.5				28	127.0	28.9	11.70	10.75	94.5	
2	5	159.3	16.6	12.9	77.5	9	29	40.0	17.6	11.70	6.06	52.3	
	6	20.5	16.6	2.9	17.5		30	36.2	27.5	11.70	2.29	18.7	
	7	30.0	16.6	2.5	15.0	10	31	100.6	33.8	12.77	12.08	94.6	
	8	159.3	16.6	12.9	77.5		32	64.0	24.9	12.77	10.47	82.9	
3	9	159.3	13.1	11.7	89.5	11	33	27.9	19.2	12.77	3.66	28.7	
	10	23.5	13.1				34	45.0	22.9	12.77	7.01	54.9	
	11	159.3	13.1	10.4	79.5	12	35	89.2	26.7	12.77	8.16	63.9	
	12	50.2	13.1	0.9	6.9		36	138.2	15.5	12.77	12.30	95.0	
4	13	159.3	22.9	21.9	95.6	13	37	73.3	34.0	12.77	8.88	64.0	
	14	14.9	22.9	2.2	9.8		38	92.0	34.9	15.44	13.98	90.6	
	15	159.3	22.1	20.8	94.1	14	39	68.4	36.5	15.44	12.53	81.2	
							40	43.5	36.0	15.44	8.60	55.7	
5	16	159.3	23.7	22.7	95.8	15	41	34.8	36.0	15.44	7.18	46.1	
	17	20.9	23.7	0.9	3.8		42	28.6	39.6	15.44			
	18	159.3	23.9	22.9	96.6	16	43	21.0	52.0	15.44	1.23	8.0	
	19	36.6	23.7	3.0	12.6		44	59.7	24.5	7.88	5.20	66.0	
6	20	159.3	23.7	22.0	93.0	11	45	147.0	26.5	7.88	7.07	89.8	
	21	159.3	24.9	23.2	93.1		46	35.3	14.5	7.88	0.92	11.7	
	22	83.6	24.5	17.9	73.0	12	47	14.6	14.8	7.88	0.83	10.5	
	23	27.0	24.5	2.6	10.6		48	46.3	31.4	11.68			
	24	159.3	24.5	22.8	93.0	19	49	23.8	23.2	11.68	2.26	19.5	
							50	59.2	22.4	11.68	7.86	68.0	

Other investigators, such as Doi (1921, *a*) and Macleod (1920), have studied the effects of low oxygen tensions on the arterial blood saturation by allowing the decerebrated experimental animal to breathe air of a lowered but constant oxygen content. Under these conditions Doi (1921, *a*) found that equilibrium was established after an exposure of from 10 to 15 minutes, and if the exposure to 14 per cent oxygen, was continued for 30 minutes, recovery of the animal was prolonged and not always possible. Adams and Morris (1921) have reported changes in the arterial blood during asphyxia produced by clamping the trachea or by plugging a bronchus. Such observations do not permit of study of the alveolar oxygen tension and are complicated by the asphyxial accumulation of carbon dioxide so that observed changes cannot be ascribed to anoxemia alone.

In the present series of experiments, animals were exposed to progressively increasing degrees of anoxemia. The exposures on the average were for periods of from 12 to 18 minutes each. The oxygen was reduced to the point at which respiration ceased in the extreme tests. The lowest oxygen content observed in the inspired air was 1.96 per cent, Experiment 14 (Table V). Recovery was rapid and complete in Experiment 14 on artificial respiration followed by natural breathing of atmospheric air. The blood pressure rapidly returned to normal. In 10 minutes from this time, respirations were reestablished and the arterial blood was found to be 94.1 per cent saturated. This speaks for the constancy of the general conditions we are striving to maintain. We observed, however, that when an animal was held for a long series of tests often blood pressure declined somewhat and other indications of poor condition slowly appeared. Under these conditions arterial unsaturation increased in comparison with the composition of the corresponding alveolar air, even at relatively high oxygen concentrations. We do not attribute this change in any way to the analgesia but rather to the position and the prolonged experimental stress. This observation does not accord with the view that oxygen secretion is in abeyance except when an animal is under extreme stress.

## DISCUSSION OF DATA.

The oxygen content of the arterial blood was found to be progressively reduced as the alveolar oxygen tension was lowered. The lowest inspired oxygen in our experiments, 1.96 percent, would correspond to an equivalent altitude of 53,800 feet or 10 miles.<sup>4</sup> The arterial blood of this dog contained 0.83 volume per cent of oxygen, a quantity but little more than the error of the method.

Bohr (1904) was the first to study the oxygen content of blood equilibrated with air of varying oxygen tensions. The study of the dissociation curve has been greatly extended since then. Dissociation curves have been published illustrating the behavior of the blood of man under varying conditions. The characteristic nature of the curves for the blood of animals of different species has also been established. Barcroft and Camis (1909) reported a curve for the dissociation of dog blood at 40 mm. carbon dioxide tension.

Because of the varying quantity of hemoglobin in different individuals the direct comparison of the oxygen content of arterial blood with the alveolar oxygen tension is not feasible unless the oxygen content of that particular blood equilibrated *in vitro* at the same tension is known. The percentage saturation of two blood samples of different hemoglobin content, however, is constant when they are equilibrated under the same conditions. The percentage saturation of the arterial blood, therefore, furnishes the most convenient basis for the comparison of data from various experiments.

The dissociation curve of dog blood determined by Barcroft and Camis (1909) was taken as the standard for the present series of experiments. This dissociation curve was established under 40 mm. carbon dioxide tension. In the majority of dogs studied by us the alveolar carbon dioxide was lower than 40 mm. Lowering of the alveolar carbon dioxide tension in the air against which a blood sample is equilibrated increases the percentage saturation slightly at any given oxygen tension. The dissociation curves for dogs blood at different carbon dioxide tensions have not been reported, so that if the curve of Barcroft and Camis is used to

<sup>4</sup> Interpolated from Humphrey's tables (Humphrey, W. J., *Physics of the air*, Philadelphia, 1920).



indicate the theoretical percentage saturation of arterial blood, some slight allowance must be made for the change in the form of the curve as a result of varying alveolar carbon dioxide tensions.

The close relationship between the dissociation curve and the arterial saturation found is striking. Table IV shows the determined percentage saturation in the arterial blood in a series of experiments on one dog. The theoretical degree of saturation calculated from the oxygen tension of the alveolar air and the dissociation curve is included for comparison. An additional ex-

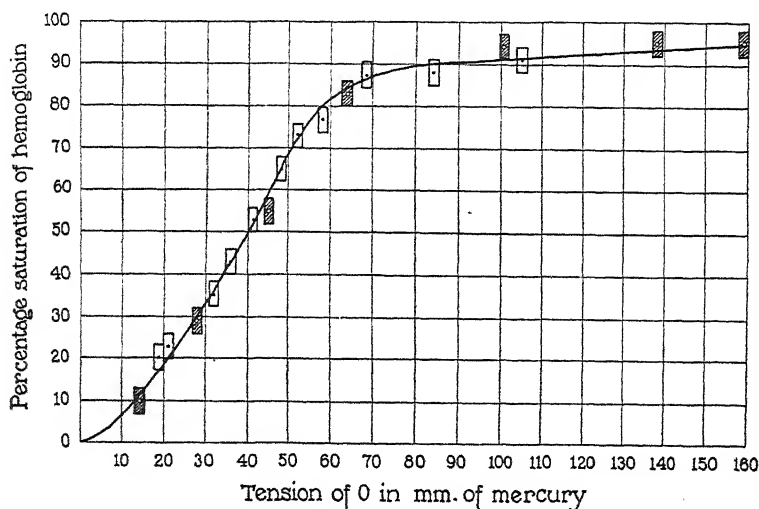


FIG. 3. Ordinates, percentage of saturation of hemoglobin. Abscissæ tension of oxygen in millimeters of mercury. Dissociation curve of dog's blood at 40 mm. tension of carbon dioxide. The plain rectangles denote dissociation determinations of Barcroft and Camis for blood *in vitro*. The cross-hatched rectangles denote determinations of arterial blood against partial pressure of alveolar oxygen *in vivo*. The area drawn around the points is the experimental error in each case.

periment on another animal is also included to show the results obtained at the lowest alveolar oxygen tension studied, 14.6 mm. This sample was drawn through the period when respiration stopped (Fig. 2).

The percentage saturation of the arterial blood is slightly less than that calculated from the dissociation curve of Barcroft and

Camis. If allowance were to be made for the different carbon dioxide tensions in the two series of experiments, this difference would be slightly, but not significantly, increased. The relationship between the dissociation curve for dog blood of Barcroft and Camis and that experimentally determined *in vivo* is most striking. This is best shown in Fig. 3 in which the individual determinations of Barcroft and Camis are plotted with the arterial blood analyses.

The two dogs that were used in the above series of experiments were in excellent condition and compensation was well maintained throughout the tests. If oxygen secretion is a factor in the compensation to anoxemia it should have been called forth by the stress of the extremely low alveolar oxygen tension obtained. Under these most favorable experimental conditions, however, the arterial oxygen approaches, but does not exceed, the maximum theoretically possible *in vitro*, as calculated from the dissociation curve. No attempt was made to calculate the diffusion pressure existing between the oxygen tension of the alveolar air and the arterial blood. Barcroft and his collaborators (1920) found between 7 and 8 mm. Douglas, Haldane, Henderson, and Schneider (1913) on Pike's Peak reported oxygen tensions in the arterial blood greater by from 7 to 44 mm. than in the alveolar air. Barcroft and Nagahashi (1921) have pointed out the difficulty of accurately determining the oxygen tension of blood that is more than 90 per cent saturated. They describe a method of equilibrating a small bubble of alveolar air with a large volume of arterial blood that gives results accurate to about 2 mm. tension. When the arterial blood is less than 90 per cent saturated, however, this method has no advantages over that used in our study. Either method would readily detect differences between the arterial and alveolar oxygen tensions of the magnitude reported by Douglas, Haldane, Henderson, and Schneider.

The first series of determinations in which only the inspired air was analyzed is given in Table V. Table VI gives the second series of experiments in which the alveolar oxygen tension was determined. Both series of determinations are shown graphically in Fig. 4. The total group of analyses shows no essential difference in behavior from the single series of experiments included in Table IV.

The arterial saturations in the first series of experiments fall slightly below those of the second series. This is to be expected, for it is recognized that at even the extremely low oxygen pressures studied the alveolar tension of necessity must still be slightly under that of the inspired air.

The determinations approach, but do not exceed, the theoretical degree of saturation established by the dissociation curve. Of the entire series of experiments but two analytical values are above the line of the dissociation curve and in both instances the difference is

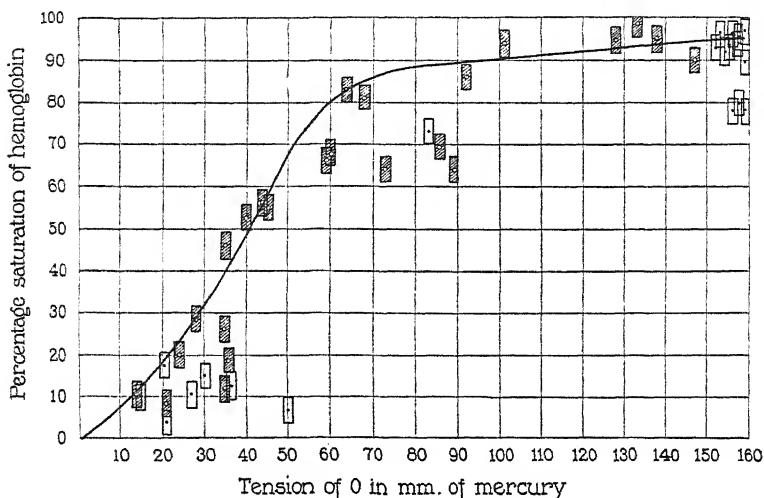


FIG. 4. Ordinates, percentage saturation of hemoglobin. Abscissæ, tension of oxygen in millimeters of mercury. Dissociation curve of dog's blood at 40 mm. tension of carbon dioxide. The cross-hatched rectangles denote determinations plotted at oxygen tension of alveolar air. The plain rectangles denote determinations plotted at oxygen tension of inspired air. The area drawn around the point is the experimental error in each case.

less than the allowable experimental error of the determination. The arterial saturation figures, on the other hand, have a marked tendency to fall below the theoretical values. This tendency emphasizes the absence of any factor of oxygen secretion in maintaining the arterial oxygen tensions. This tendency also emphasizes the necessity for the efficient operation of all the factors of physical compensation in order to maintain the maximal satura-

tion of the arterial blood. Any lowering in the efficiency of the compensating mechanism, either in the circulatory rate, circulatory volume, respiratory volume, or the completeness of pulmonary ventilation is immediately expressed in a larger unsaturation value of the corresponding arterial blood. The difference between the alveolar and arterial oxygen tensions is always augmented under such conditions.

If oxygen secretion should occur it is possible that it might, in part at least, be under nervous control. Any secretory fibers to the lungs would be located in the vagi and section of these nerves should affect the oxygenation of the arterial blood. This experiment was tested in one animal (Dog 11, Experiment 47, Table IV). The response of this dog to anoxemia was no different from that of the others in the experimental series. The degree of saturation of the arterial blood was not affected by the section of the vagi.

TABLE VI.

*Arterial Blood Equilibrated with Alveolar Air.*

Experiment 25, Sept. 26, 1921. Dog 7.

	Oxygen in alveolar air.		Oxygen in arterial blood.	
	Per cent.	Tension.	Volumes per cent.	Saturation.
		mm.		per cent
Before equilibration.....	11.54	85.4	10.5	64.5
After " .....	10.38	77.3	10.8	66.2

Barcroft (1920) found that when arterial blood from a man, living under a reduced oxygen tension, was equilibrated with a corresponding sample of alveolar air, the blood absorbed oxygen from the air. We have confirmed this finding in the dog through the range of anoxemia studied. Because of the construction of the tonometer it was difficult to saturate the blood sample completely, but oxygen was always absorbed by the blood sample and never the reverse. This point is vital to the present experiments.

The present day advocacy of the theory of oxygen secretion is based on data obtained by the indirect carbon monoxide method. This method assumes the stability of the carbon monoxide-hemoglobin compound. Haggard and Henderson (1921) have recently

shown that carbon monoxide in the blood may rapidly be liberated from the hemoglobin fixation, particularly under conditions of an excess of carbon dioxide in the blood. Without attempting a detailed discussion of the method we may emphasize the great advantage of a direct over an indirect method.

In our experiments, the percentage saturation of the arterial blood was directly determined under conditions of progressively induced anoxemia. The proponents of the secretory theory have assumed that the capacity for oxygen secretion exists but is called forth only during extreme oxygen lack. The conditions of our experiments would seem to be adequate to call forth this mechanism if present. No evidence of such a mechanism was found. Nowhere have we obtained evidence of an arterial oxygen tension higher than that in the alveolar air. The current conception of a purely physical mechanism regulating the passage of oxygen though the pulmonary epithelium is confirmed for all stages of anoxemia.

#### SUMMARY.

The lowering of the percentage saturation of the arterial blood in dogs breathing air progressively reduced in oxygen tension closely parallels the dissociation curve determined *in vitro* by Barcroft and Camis.

No evidence was obtained to indicate the presence of a higher oxygen tension in the arterial blood than was present in the alveolar air.

When alveolar air was exposed to a simultaneous sample of arterial blood, oxygen was absorbed by the blood.

Section of the vagi was without effect on the degree of saturation of the arterial blood during progressive anoxemia.

Experimental animals must be maintained in a state of high circulatory and respiratory efficiency in order to secure the maximal degree of saturation of the arterial blood.

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A QUANTITATIVE STUDY OF THE ADSORPTION IN SOLUTION AND AT INTERFACES OF SUGARS, DEXTRIN, STARCH, GUM ARABIC, AND EGG ALBUMIN, AND THE MECHANISM OF THEIR ACTION AS EMULSIFYING AGENTS.

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There is a wide divergence in opinion at the present time as to what the real mechanism of the action of colloid protectors and emulsifying agents may be. While it is generally known that the same substances may be used as protectors of very finely divided solid precipitates by preventing agglomeration, and as emulsifying agents by stabilizing liquid droplets dispersed in a liquid medium, and that the most efficient of these substances are themselves peptized colloids, it is by no means generally agreed that the mechanism of the two processes is the same. On the other hand there have been numerous attempts to embody in a single generalization the properties and conditions necessary for colloid peptization and protection, and for emulsification. Thus Plateau<sup>1</sup> and Quincke<sup>2</sup> without understanding the true nature of adsorption considered that the most important factor required in an emulsifying agent was that it should lower the surface tension of one of the liquids though they also realized the large influence of viscosity. Pickering<sup>3</sup> presented the theory that the emulsifying agent must form small insoluble particles to cover the droplets. Fischer<sup>4</sup> believed that ability to combine with water is the most essential

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<sup>1</sup> Plateau, J., *Ann. Physik.*, 1870, cxli, 44.

<sup>2</sup> Quincke, G., *Ann. Physik.*, 1888, cclxxi, 580.

<sup>3</sup> Pickering, S. U., *J. Chem. Soc.*, 1907, xci, 2001.

<sup>4</sup> Fischer, M. H., and Hooker, M. O., *Science*, 1916, xliii, 468.



attribute. Bancroft<sup>5</sup> states that an emulsifying agent is adsorbed into the interface between dispersed phase and dispersing medium and produces a coherent film which by its viscous nature prevents coalescence of the particles. He points out that while most emulsions are made with gelatinous colloids as emulsifying agents, the same purpose would be served by any substance which will be adsorbed to the interface and make it viscous. Similarly the mechanism of stabilizing or protecting colloids is to be explained by adsorption of the agent which is either soluble in water or is solvated by water.<sup>6</sup> Holmes,<sup>7</sup> in working with gelatin as an emulsifying agent, could get no evidence of any concentration of gelatin by adsorption around oil droplets or that as oil content is increased the gelatin content must be increased as would be the case if adhesion layers were formed. He considers that the leading factor is simply viscosity, not the maximum but the "most favorable." These results are in direct contradiction to the work of Winkelblech<sup>8</sup> who shook a dilute solution of gelatin with benzene and found a film of gelatin at the dineric interface, a phenomenon which he proposed as a method of analysis for gelatin. He declared that the adsorption of the emulsifying agent at the interface was inversely proportional to the ease of peptization of the substance by one of the liquids in the emulsion. In a paper before the American Chemical Society at Rochester, N. Y., April 1921, Holmes showed that the formation of an adsorbed film was the principal factor in the use of cellulose nitrate.

It is at once apparent that quantitative data on the action of peptizing colloids and related substances is almost completely lacking, and that satisfactory conclusions cannot be drawn without such data. The object of the present research was, therefore, to study by the most accurate known methods the behavior of five substances which, to a greater or less extent, act as colloid protectors or emulsifying agents, *viz.* sugars, dextrin, starch, gum arabic, and egg albumin, throughout the greatest possible range of concentration, each alone and, for the purpose of studying ion

<sup>5</sup> Bancroft, W. D., *J. Phys. Chem.*, 1913, xvii, 501; Applied colloid chemistry; general theory, New York, 1921, 225.

<sup>6</sup> Bancroft, <sup>5</sup> p. 225.

<sup>7</sup> Holmes, H. N., and Child, W. C. J., *Am. Chem. Soc.*, 1920, xlii, 2049.

<sup>8</sup> Winkelblech, K., *Z. angew. Chem.*, 1907, xix, 1953.

adsorption, in the presence of the following electrolytes: hydrochloric acid, sodium hydroxide, iodide, sulfate, and bicarbonate, as respectively, acid, base, liquefying or peptizing salt, precipitating or peptizing salt, and a biologically significant substance. It is, of course, unnecessary to point out the manifold application of such data to the fields of adsorption and digestion in physiology, many industrial processes including the manufacture of soaps, paints, medical preparations, oils, inks, and numerous other products, flotations, sewage disposal, electroplating—in short, in every case where minute “drops, bubbles, grains, filaments or films” require stabilization.

#### EXPERIMENTAL METHODS.

Each dilution of each one of the five substances under investigation, alone and in the presence of each of the five electrolytes, was studied under the following heads: (a) Surface tension at 25°. (b) Interfacial tension between solution and benzene, and in some cases caprylic alcohol for purposes of comparison between aromatic hydrocarbon and long chain alcohol. (c) Viscosity. (d) Density. (e) Actual observations upon emulsions made up uniformly with benzene and kerosene and studied for heaviness of creaming and stability as measured by time of existence.

The surface tension of the solutions was determined by the drop, weight method, using a Morgan drop-weight apparatus<sup>9</sup> with calibrated<sup>10</sup> tip with radius of 0.2673 cm., and the values calculated by

the use of the Harkins and Brown<sup>11</sup> formula:  $\gamma = \frac{mg}{2\pi r} \psi\left(\frac{r}{V^{\frac{1}{3}}}\right)$ ,

where  $\gamma$  is the surface tension in dynes per cm.,  $m$  the weight of a drop,  $g$  the acceleration due to gravity,  $r$  the radius of the tip,  $V$  the molecular volume determined from the density, and  $\psi$  the function corresponding to the fraction of an ideal drop which actually falls. There can be no criticism of the accuracy of this method as shown by the recent confirmatory results of Richards and Carver<sup>12</sup> in their critical study of the capillary height method.

<sup>9</sup> Morgan, J. L. R., *J. Am. Chem. Soc.*, 1911, xxxiii, 349.

<sup>10</sup> Harkins, W. D., and Humphrey, E. C., *J. Am. Chem. Soc.*, 1916, xxxviii, 228.

<sup>11</sup> Harkins W. D., and Brown, F. E., *J. Am. Chem. Soc.*, 1919, xli, 515.

<sup>12</sup> Richards, T. W., and Carver, E. K., *J. Am. Chem. Soc.*, 1921, xliii, 827.

TABLE I.  
Effect of Emulsifying Agents on Benzene-Water and Kerosene-Water Emulsions.

Oil phase.	Concen- tration of agent.	Electrolyte.	Condition of emulsion.															
			Beginning.				After 1 week.				After 4 weeks.				After 7 weeks.			
			Sucrose.	Dextrin.	Starch.	Gum arabic.	Egg albumin.	Sucrose.	Dextrin.	Starch.	Gum arabic.	Egg albumin.	Sucrose.	Dextrin.	Starch.	Gum arabic.	Egg albumin.	
Benzene.....	per cent																Egg albumin.	
Benzene.....	0	0	3					1									Gum arabic.	
Kerosene.....	0	0	3					2									Gum arabic.	
Benzene.....	0.01	0	4	5	4	6		1	2	3	5		1	2	5		Gum arabic.	
Kerosene.....	0.01	0	3	6	9	8		3	4	9	6		3	4	9	6	Gum arabic.	
Benzene.....	0.1	0	4					2									Gum arabic.	
Kerosene.....	0.1	0	3					2									Gum arabic.	
Benzene.....	0.918	0					10										Egg albumin.	
Kerosene.....	0.918	0					10										Egg albumin.	
Benzene.....	1.0	0	3	5	6	8		2	4	6	5		1	3	6	5	Egg albumin.	
Kerosene.....	1.0	0	3	5	9	8		3	3	9	4		3	2	9	3	Egg albumin.	
Benzene.....	10.0	0	4	8		9		2	6	9	9		1	6	9	9	Egg albumin.	
Kerosene.....	10.0	0	8	6		9		6	3		9		5	3		9	Egg albumin.	
Benzene.....	30.0	0	5					3					2				Egg albumin.	
Kerosene.....	30.0	0	5					4					4				Egg albumin.	
Benzene.....	0.005	HCl	4	2				0	0				—	—			Egg albumin.	

In the table 0 indicates Disappeared; 1, Traces; 2, Extremely slight; 3, Very slight; 4, Slight; 5, Fair; 6, Good; 7, Very good; 8, Heavy; 9, Very heavy; 10, Excellent.

Kerosene.....	0.005	HCl	4	4	4	0	2	2	4	6	4	2	2	4	6	10
Benzene.....	0.05	HCl	4	4	4	0	0	0	0	0	0	—	—	—	—	10
Kerosene.....	0.05	HCl	4	4	4	0	0	0	0	0	0	—	—	—	—	10
Benzene.....	0.5	HCl	3	4	4	0	4	5	6	4	6	2	2	4	4	10
Kerosene.....	0.5	HCl	4	4	4	0	2	5	6	4	6	2	2	4	4	10
Benzene.....	0.439	HCl				10										10
Kerosene.....	0.439	HCl				10										10
Benzene.....	5.0	HCl	2	6	6	2	5	6	6	6	6	1	5	6	—	10
Kerosene.....	5.0	HCl	4	4	4	2	6	0	0	0	0	—	—	—	—	10
Benzene.....	0.005	NaI	4	4	4	0	0	0	0	0	0	—	—	—	—	10
Kerosene.....	0.005	NaI	4	4	4	0	2	0	0	0	0	—	—	—	—	10
Benzene.....	0.05	NaI	3	3	3	0	0	0	0	0	0	—	—	—	—	10
Kerosene.....	0.05	NaI	3	3	3	0	0	0	0	0	0	—	—	—	—	10
Benzene.....	0.5	NaI	3	4	4	0	4	6	6	6	6	2	6	6	6	10
Kerosene.....	0.5	NaI	3	4	4	0	2	4	4	4	4	2	1	1	6	10
Benzene.....	0.459	NaI	3	4	4	10										10
Kerosene.....	0.459	NaI				10										10
Benzene.....	5.0	NaI	2	6	5	2	4	4	4	4	4	1	3	4	4	10
Kerosene.....	5.0	NaI	5	5	5	2	4	4	4	4	4	2	4	2	4	10
Benzene.....	0.005	NaOH	4	4	4	2	2	2	2	2	2	2	0	1	5	10
Kerosene.....	0.005	NaOH	5	5	5	2	4	4	4	4	4	1	2	2	2	10
Benzene.....	0.05	NaOH	2	2	2	10+										10
Kerosene.....	0.05	NaOH	5	5	5	10+										10
Benzene.....	0.5	NaOH	2	4	4	7	2	2	2	5	1	2	2	2	1	10
Kerosene.....	0.5	NaOH	5	5	5	4	2	2	2	2	2	1	2	2	1	10
Benzene.....	0.459	NaOH				10+										10
Kerosene.....	0.459	NaOH				10+										10
Benzene.....	5.0	NaOH	2	5	5	10+										10
Kerosene.....	5.0	NaOH	5	5	5	10+										10

TABLE I—Concluded.

Oil phase.	Concentration of agent.	Electrolyte.	Condition of emulsion.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
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			Sucrose.	Dextrin.	Starch.	Gum arabic.	Egg albumin.	Sucrose.	Dextrin.	Starch.	Gum arabic.	Egg albumin.	Sucrose.	Dextrin.	Starch.	Gum arabic.	Egg albumin.	Sucrose.	Dextrin.	Starch.	Gum arabic.	Egg albumin.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			
Benzene.....	0.005	NaHCO <sub>3</sub>	4	2				0	0				—	—				—	—																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						

The interfacial tensions were measured by the corresponding drop-volume method using a modified form of the apparatus described by Harkins and Humphrey<sup>13</sup> with the correction curve for calculation. Two or more runs were made in each case to insure accurate checking.

The relative viscosities were measured by means of a special Ostwald type viscosimeter and with the following precautions: constant temperature throughout; same treatment of the solution as regards shaking and previous treating; recognition of the large effect of viscosity changes in emulsoids with time so that runs were made only after the emulsoids were allowed to stand for some time, and at the same time that surface tension measurements and actual emulsions were made. In most cases, of course, the emulsoids were so dilute that a practically constant viscosity was observed, agreeing with the observations of von Schroeder.<sup>14</sup>

Density measurements were particularly important for the exact calculation of surface tension. They were made at 25° with pycnometer or carefully calibrated Westphal balance.

The emulsions were all made up in the same manner, with the same relative volumes, 75 per cent oil to 25 per cent solution or emulsoid. The benzene was dehydrated and distilled over metallic sodium at 80–82°. Kerosene was purified and dehydrated over metallic sodium. The emulsion was made by adding 30 cc. of oil in 10 cc. portions to 10 cc. of the solution. After each addition of oil the bottles (glass-stoppered specimen bottles of uniform size) were shaken a full 10 minutes or for all three additions 4,800 strokes. These were then placed in a quiet place, or in some cases centrifuged, and observed after various lengths of time. The results are tabulated together in Table I in which the emulsions are classified on a scale of ten from the standpoint of efficiency of emulsification.

#### EXPERIMENTAL RESULTS.

##### *Sugars.*

Most of the work on sugars was conducted with purest sucrose. While solutions of sugar are not colloidal or gelatinous in the sense

<sup>13</sup> Harkins and Humphrey,<sup>10</sup> p. 236.

<sup>14</sup> von Schroeder, P., *Z. physik. Chem.*, 1903, xlv, 75

that those of dextrin, starch, gum arabic, and egg albumin are, this non-electrolyte is of great theoretical importance, although it may not be considered nearly so effective a colloid protector or emulsifying agent as the emulsoids. Sugar is usually considered to be molecularly dispersed in aqueous solutions but Lobry de Bruyn and Wolff<sup>15</sup> have shown that concentrated solutions show the Tyndall phenomenon characteristic of a colloid. It is interesting, therefore, to discover if this tendency may account for such powers as an emulsifying agent as sugar solutions may possess. Surprisingly little work has been done with sugar solutions and none quantitatively. There are, of course, numerous cases where strong sugar solutions prevent the formation of visible solid precipitates, such as calcium silicate,<sup>16</sup> silver chromate, silver chloride,<sup>17</sup> lime,<sup>18</sup> and hydrous oxides of copper, uranium, and iron.<sup>19</sup> Bancroft<sup>20</sup> states that no work has been done on the direct peptization of precipitates by such a dissolved non-electrolyte. Grimaux<sup>21</sup> found that invert sugar was seven times as effective as cane-sugar in holding up ferric oxide. Bancroft<sup>6</sup> considers that sols are stabilized by non-electrolytes such as sugar by very strong adsorption of the sugar so that the colloid will behave as though water-soluble. There is no data cited on this point however. It would necessarily follow from this idea that sugar could act as an emulsifying agent if it will go into the interface of an oil-water mixture and make it sufficiently viscous. On the other hand if molecular dispersion in water solution is considered as the maximum limiting case of peptization no concentration of sugar would be found in the interface in keeping with the general rule that the amount adsorbed at the interface is inversely proportional to the ease of peptization by one of the liquids. The above references constitute the present knowledge of the action of sugar. Aside from limited data such as may be found in Landolt-Börnstein tables on the surface tension of sugar solutions, there is nothing quantitative.

<sup>15</sup> Lobry de Bruyn, C. A., and Wolff, L. K., *Rec. trav. chim. Pays-Bas*, 1904, xxiii, 155.

<sup>16</sup> Weisberg, J., *Bull. Soc. Chim.*, 1896, xv, series 3, 1097.

<sup>17</sup> Lobry de Bruyn, C. A., *Ber. chem. Ges.*, 1902, xxxv, 3079.

<sup>18</sup> Graham, T., *J. Chem. Soc.*, 1862, xv, 253.

<sup>19</sup> Riffard, E., *Compt. rend. Acad.*, 1873, lxxvii, 1103.

<sup>20</sup> Bancroft,<sup>5</sup> p. 168.

<sup>21</sup> Grimaux, E., *J. Chem. Soc.*, 1884, xlv, 966.

The experimental values of cane-sugar solutions on specific gravity, relative viscosity, surface tension, and interfacial tension against benzene are tabulated in Table II. While in all cases the values of  $\gamma$  are above those of pure water there is a very interesting variation discovered in the dilute solutions. There is a gradual slight rise above 72.05 dynes for pure water to 0.01 per cent solutions, followed by a *decrease* to 0.1 per cent and another increase at somewhat less than 1 per cent which continues up to the most concentrated solution, 35 per cent. It will be shown later that the same rise and depression in  $\gamma$  for very dilute solutions of the four emulsoids, dextrin, starch, gum arabic, and egg albumin, is observed, although the second ascent is not observed for them. The peculiar behavior for sucrose, which in solution seems to be uniformly negatively adsorbed, is difficult to explain, though it may be related to the hydration of the sugar molecules, agglomeration, or both.

The values in the table show at a glance that, except in the cases of dilute solutions and of HCl, the presence of the electrolytes enhances the value of  $\gamma$ . In 0.005 per cent solutions made 0.5 M with the electrolytes, hydrochloric acid and sodium iodide have no effect, but sodium hydroxide *lowers*  $\gamma$ , to a considerable extent. Thus, though 0.005 per cent sucrose has a value of 72.13 and the hydroxide alone of 72.60, the solution of both is 71.77, *even below that of pure water*. This can be explained only upon the basis of chemical reaction in very dilute solutions or a very specific adsorption of hydroxyl ions. It will be shown that dextrin and starch act in exactly the same way. In the more concentrated solutions the hydroxide acts similarly to the sulfate and bicarbonate. Except in the case of the 0.1 per cent solution which marks the maximum point of deflection, hydrochloric acid, which alone has practically no effect upon the surface tension of water, lowers the value of the sugar solution. The specific effect of the iodide ion which is so markedly adsorbed and peptizing upon emulsoids is even here quite apparent. This entire study is one of great interest inasmuch as two factors enter in: the effect of electrolytes on sugar solutions and of sugar solutions on the electrolytes. The variations are to be accounted for by differences in adsorption of ions upon sugar molecules and of water upon both sugar and the ions. It should be noted that 0.5 per cent sugar solutions, though



TABLE II.  
*Sugar (Sucrose).*

Concentration of sucrose by weight.	Electrolyte.	Specific gravity.	Relative viscosity.	Surface tension.	Surface tension at interface.*
<i>per cent</i>				<i>dynes per cm.</i>	<i>dynes per cm.</i>
None.	H <sub>2</sub> O	0.9970	1.000	72.05	34.28
"	HCl	1.0068	1.041	72.09	34.47
"	NaI	1.0525	1.020	72.86	34.82
"	NaOH	1.0210	1.117	72.60	34.70
"	NaHCO <sub>3</sub>	1.0267	1.123	73.04	34.65
"	Na <sub>2</sub> SO <sub>4</sub>	1.0601	1.245	73.36	36.16
0.005†	None.		1.003	72.16	34.49
0.005	HCl	1.0072	1.045	72.15	34.46
0.005	NaI	1.0532	1.025	72.14	35.14
0.005	NaOH	1.0211	1.118	71.77	35.00
0.005	NaHCO <sub>3</sub>	1.0268	1.122	73.49	35.24
0.005	Na <sub>2</sub> SO <sub>4</sub>	1.0661	1.223	73.52	36.72
0.01	None.	0.9972	1.005	72.27	34.68
0.05	"		1.011	72.20	34.74
0.05	HCl	1.0079	1.051	72.27	34.80
0.1	None.	0.9998	1.017	72.14	34.79
0.5	"		1.028	72.31	34.03
0.5	HCl	1.0087	1.049	72.18	34.33
0.5	NaI	1.0544	1.026	72.85	34.60
0.5	NaOH	1.0228	1.129	72.97	35.08
0.5	NaHCO <sub>3</sub>	1.0294	1.140	72.80	35.35
0.5	Na <sub>2</sub> SO <sub>4</sub>	1.0737	1.235	73.34	36.39
1.0	None.	1.0024	1.039	72.48	35.57
5.0	"		1.174	72.57	33.80
5.0	HCl	1.0276	1.185	72.50	34.57
10.0	None.	1.0377	1.328	72.69	33.97
10.0	HCl	1.0489	1.389	72.59	34.67
15.0	None.		1.482	72.90	33.94
15.0	HCl	1.0727	1.677	73.10	34.07
15.0	NaI	1.1170	1.669	73.14	34.27
15.0	NaOH	1.0833	1.939	73.41	34.87
15.0	NaHCO <sub>3</sub>	1.0905	1.886	73.45	34.22
15.0	Na <sub>2</sub> SO <sub>4</sub>	1.1237	2.106	74.32	36.51
20.0	None.	1.0805	1.636	73.11	33.90
30.0	"	1.1257	3.030	73.48	33.54
35.0	"	1.1510	4.098	73.58	32.85

\* Determinations made with 90 per cent benzene, sp. gr. 0.8715.

† Values for sucrose solutions without electrolytes, percentage concentrations 0.005, 0.05, 0.5, 5.0, and 15, taken from curves. All other values in the table are actual determinations.

possessing a higher surface tension than 0.01 per cent, produce a marked lowering of the surface tension of sodium bicarbonate, although the 0.005 per cent raises it, and that sodium sulfate in 0.5 per cent sugar solution has the same value as if it were present in pure water. The bicarbonate has the same surface tension both in 0.005 and 15 per cent sugar solutions.

The effect of the presence of hydrochloric acid in solutions of sucrose is particularly interesting because of inversion with time. Inversion has not before been followed by means of possible variations in surface tension with time. In Table III are some representative data. It is at once apparent that the changes are exceedingly small though generally there is a slight increase which has been confirmed by repeated observations. The small structural effect is shown by the fact that a 1 per cent sugar solution made 0.5  $M$  with hydrochloric acid has a surface tension of 72.18 upon mixing, and 72.26 after inversion, while a 1 per cent levulose solution with acid is 72.28, a 1 per cent dextrose solution with acid is 72.25, and a 1 per cent equal mixture of levulose and dextrose with acid is 72.25. Absolutely no evidence has been obtained to confirm the statement of Grimaux<sup>21</sup> that invert sugar is seven times as efficient in holding up suspensoids or emulsoids as sucrose. In equal concentrations no difference has been detected in any case.

Considering the surface tension values at the interface between sugar solutions and benzene, very interesting results are obtained. Starting with the value of 34.28 for the interfacial tension of water-benzene and adding sugar, there is at first an increase to 0.1 per cent solutions, then a sharp decrease, another increase between 1 and 10 per cent solutions, an almost constant value between 10 and 20 per cent, and a final continual decrease with increase in concentration up to 35 per cent. Even with these variations, all sugar solutions above 1 per cent in concentration, lower the interfacial tension between water and benzene, a result which is apparently favorable for the action of sugar as an emulsifying agent. Since sugar does not lower the surface tension of water alone and is seemingly negatively adsorbed in the system solution-vapor, it at once follows that the positive effect at the benzene interface must be specific for the benzene at the higher concentrations of sugar. That this is true is quite clearly shown by the fact that at the interface between water and caprylic alcohol, an increase

in sugar *increases* the tension, following closely the action upon water alone. This is shown in Table IV. It must be concluded,

TABLE III.

*Sugar—Effect of Inversion of Sucrose on Surface Tension, Interfacial Surface Tension, and Viscosity.*

Concentration of sugars by weight.	Acid.*	Specific gravity.†	Relative viscosity.	Surface tension.		Interfacial surface tension.‡	
				Fresh.	After 24 hours.	Fresh.	After 24 hours.
Sucrose.							
<i>per cent</i>				<i>dynes per cm.</i>	<i>dynes per cm.</i>		
0.005	None.		1.003	72.16		34.49	
0.005	HCl	1.0072	1.045	72.15	72.29	34.46	34.33
0.05	None.		1.011	72.20		34.74	
0.05	HCl	1.0079	1.051	72.27	72.22	34.80	34.73
0.5	None.		1.028	72.31		34.03	
0.5	HCl	1.0087	1.049	72.18	72.26	34.33	34.40
5.0	None.		1.174	72.57		33.80	
5.0	HCl	1.0276	1.185	72.50	72.63	34.57	34.82
10.0	None.	1.0377	1.328	72.69		33.97	
10.0	HCl	1.0489	1.389	72.59	72.76	34.67	34.89
15.0	None.		1.482	72.90		33.94	
15.0	HCl	1.0727	1.677	73.10	73.27	34.07	34.68
Levulose.							
1.0	None.	1.0022	1.023	72.18		33.57	
0.5	HCl	1.0088	1.047	72.28		34.50	
Dextrose.							
1.0	None.	1.0019	1.026	72.29		34.12	
0.5	HCl	1.0089	1.043	72.25		34.82	
Levulose and dextrose.							
1.0	None.	1.0015	1.025	72.30		34.17	
0.5	HCl	1.0087	1.044	72.25		34.62	

\* 0.5 M HCl.

† With solutions fresh.

‡ Determinations made with 90 per cent benzene, sp. gr. 0.8715.

therefore, that the chemical nature of the oil used in making an emulsion will determine, from the standpoint at least of surface energy relationships, the efficiency of sugar as an emulsifying

agent. It will be shown later that kerosene forms much better emulsions in the presence of sugar than benzene following a greater decrease of the interfacial tension kerosene-water.

All of the electrolytes increase the interfacial tension values of sugar solution-benzene, following in a somewhat parallel way the variations of sugar concentration. Here again the expected does not occur, *e.g.* that HCl should lower the values as in the case of solution-vapor. Obviously, interpretations are not justified until experiments varying the concentration of electrolyte with given sugar content are made.

TABLE IV.  
*Caprylic Alcohol.*

Surface tension = 26.35. Relative viscosity = 3.126. Density = 0.82026.

Solution.	Concentration by weight.	Surface tension at interface with caprylic alcohol.	Surface tension at interface with 90 per cent benzene.
	<i>per cent</i>	<i>dynes per cm.</i>	<i>dynes per cm.</i>
Water.....	None.	9.80	34.28* 33.72†
Sucrose.....	30.0	10.88	33.54
Dextrin.....	10.0	3.85	36.41
Starch.....	1.0	10.17	35.10
Gum arabic.....	10.0	9.24	22.87

\* Determination made with 90 per cent benzene, sp. gr. 0.8715, used with sucrose.

† Determination made with 90 per cent benzene, sp. gr. 0.8745, used with dextrin, starch, and gum arabic.

Parallel in importance with the effect of sugar upon interfacial tension is the viscosity effect. It has already been pointed out that the view of Bancroft is that any substance which is *adsorbed to an interface and is sufficiently viscous* will act as an emulsifying agent while Holmes found that *favorable viscosity* alone may account for such an action. Of course, the viscosity of sugar, a molecular dispersoid, increases in a regular way with concentration. Electrolytes increase viscosity slightly in dilute solutions but produce a very great lowering in the more concentrated solutions. It is noteworthy that this effect is not only observed with sugar, a molecular dispersoid, but also with the emulsoids.

Freundlich<sup>22</sup> has shown that increase in viscosity in any case goes hand in hand with increase in agglomeration which in turn involves decrease in the amount of available free water. Another type of agglomeration, however, may be accompanied by a decrease in viscosity. This point will be fully discussed under dextrin and starch. It would seem, therefore, that the electrolytes prevent the agglomeration of sugar in higher concentrations. On the other hand the real effect may be of the sugar acting on the electrolytes. The well grounded fact that ions are more strongly hydrated than electrically neutral undissociated molecules explains why increase in dissociation and increase in viscosity go hand in hand. As a result of the magnetic field about the charged particles the ions may very strongly adsorb the solvent—a phenomenon which would be greatly hindered in the presence of adsorbed sugar probably. In justification of this it is, of course, well known that viscous sugar or glycerol solutions have a marked effect on ionic conductivity, though equally viscous solutions of such substances as gelatin, agar, and proteins have practically no hindrance whatever to the motion of ions and crystalloid molecules.<sup>23</sup> Here again interpretations must be incomplete until observations have been made varying the concentration of electrolyte. Suffice it to say that below 20 per cent solutions the electrolytes increase viscosity while above 20 per cent they lower it. If viscosity were a predominating factor, therefore, it would be expected that the best emulsions would be obtained in the presence of electrolytes, particularly sodium sulfate, below 20 per cent, and with pure sugar solutions above 20 per cent, and that if the maximum viscosity is the “most favorable” the very best emulsions would be obtained with 35 per cent sugar solution without electrolytes. Such is found to be the case if the rating of the emulsions in Table I is considered. But it should also be remembered that 35 per cent sugar solution without electrolytes *produces the maximum lowering of interfacial tension* between water and either benzene or kerosene, and, therefore, there are two superimposed favorable effects for the emulsifying action of sugar. There is no question, however, that viscosity is the more important of the two factors,

<sup>22</sup> Freundlich, H., *Tr. Faraday Soc.*, 1913, ix, 66.

<sup>23</sup> Cf. Walden, P., *Z. physik. Chem.*, 1906, iv, 209.

for the stability of the emulsions follows more nearly the increase in viscosity aided both by increase in concentration and by presence of electrolytes in the more dilute solutions, than it does the changes in adsorption as measured by interfacial tension. Thus emulsions of caprylic alcohol in water are increasingly better with increase in viscosity of the sugar solution added, although the interfacial tension is also gradually increased. While for benzene the maximum viscosity is also the most favorable, this is not true with kerosene emulsions. A 10 per cent sugar solution produces much better emulsions than 30 per cent solutions, paralleling the discovery of Holmes that too viscous gelatin solutions were not desirable for emulsions of the same oil.

By a consideration of sugar alone, therefore, it is apparent that no general rule for the mechanism of the action of emulsifying agents can be laid down, nor can any one general rule be enunciated for the action of one agent under all conditions. Much depends upon the chemical nature of the liquids being emulsified. With sugar the primary attribute is viscosity—usually maximum—even though there is evidence also of adsorption at higher concentrations upon the emulsoid particles so that the colloid tends to act like a water-soluble one. On the other hand viscosity cannot be considered the only prime requisite of an emulsifying agent; for though sugar furnishes a large and favorable viscosity, it does not compare as an emulsifying agent with even minute concentrations of other substances in which the viscosity factor is largely negligible.

#### *Dextrin and Starch.*

These two substances will be considered together because of their close relation. Added interest is placed in these substances as a result of the recent work of Herzfeld and Klinger<sup>24</sup> who declare that dextrin is not a split-product of starch at all but simply a more highly dispersed starch. Thus the blue color of iodine adsorbed upon starch and the brown color upon dextrin is merely a result of the greater dispersion in the latter case. Diastatic action, is, like dextrinization of starch by simple adsorption of the surface by solutions, merely a process of alteration of the degree of disper-

<sup>24</sup> Herzfeld, E., and Klinger, R., *Biochem. Z.*, 1920, cvii, 268.

sion to form a large increase in surface which is an essential preparation for later hydrolysis. If then this view be true, the transition from starch through soluble starch (more highly dispersed) to dextrin (most highly dispersed colloiddally) should be a continuous one,<sup>25</sup> and the differences in the emulsifying action of dextrin, soluble starch, or ordinary starch should be related merely to the degree of dispersion of the emulsoid. The relations are further complicated, however, by the graduations between molecular solubility in extremely dilute solutions, the suspensoid state in dilute solutions, and emulsoid state at higher concentrations. This will be considered from the standpoint of viscosity. The whole question is one of great importance biologically, inasmuch as animal glycogen is really dextrinized or highly dispersed starch and quantitative measurements should throw considerable light upon its action in living organisms.

Dextrin and soluble starch are water-peptizable colloids and consequently serve to act as colloid protectors<sup>25</sup> and emulsifying agents. Zsigmondy<sup>26</sup> has determined the gold number (number of mg. required to prevent the color change in a 10 cc. red gold solution containing 0.0053 to 0.0058 per cent gold, when 1 cc. of a 10 per cent solution of sodium chloride is added) of dextrin as 10 to 20 and of potato starch as 25. Winkelblech<sup>8</sup> pointed out that soluble starch formed a film at dineric interfaces because of the difficulty of peptization by water. Friedenthal<sup>27</sup> found that soluble starch exhibited a slight depression of the freezing point and a molecular weight of 9,450 while ordinary starch has a molecular weight of at least 32,400.<sup>28</sup>

Little work has been done on surface tension. Ostwald<sup>29</sup> states that gelatinizing colloidal mixtures have a lower surface tension than their pure dispersion medium. In Table V are shown the surface tension relationships of dextrin and in Table VI, those of soluble starch together with values for ordinary starch. The close

<sup>25</sup> Lachaud, M., *Bull. Soc. Chim. de Paris.*, 1896, xv, series 3, 1105.

<sup>26</sup> Zsigmondy, R., *Z. anal. Chem.*, 1901, xl, 697.

<sup>27</sup> Friedenthal, H., *Centr. Physiol.*, 1898-99, xii, 849.

<sup>28</sup> Brown, H. T., and Morris, G. H., *J. Chem. Soc.*, 1888, liii, 610.

<sup>29</sup> Ostwald, W., *A handbook of colloid chemistry; The recognition of colloids, the theory of colloids, and their general physico-chemical properties*, translated by Fischer, M. H., Philadelphia, 2nd edition, 1919, 51.

similarity is very marked in the more dilute solutions. In both cases there is a gradual rise in surface tension above that of pure water until a 0.01 per cent solution is reached, followed by a regular decrease to *exactly the same degree*. Thus 1 per cent dextrin has a value for  $\gamma$  of 71.98 and 1 per cent starch of 71.96. At higher

TABLE V.  
*Dextrin.*

Concentration of dextrin by weight.	Electrolyte.	Specific gravity.	Relative viscosity.	Surface tension.	Surface tension at interface.*
<i>per cent</i>				<i>dynes per cm.</i>	<i>dynes per cm.</i>
None.	H <sub>2</sub> O	0.9970	1.000	72.05	33.72
"	HCl	1.0068	1.041	72.09	34.38
"	NaI	1.0525	1.020	72.86	34.55
"	NaOH	1.0210	1.117	72.60	34.97
"	NaHCO <sub>3</sub>	1.0267	1.123	73.04	34.37
"	Na <sub>2</sub> SO <sub>4</sub>	1.0601	1.245	73.36	34.50
0.005†	None.		1.0145	72.23	34.16
0.005	HCl	1.0076	1.085	71.96	34.45
0.005	NaI	1.0512	1.025	72.31	34.45
0.005	NaOH	1.0202	1.120	72.67	35.13
0.005	NaHCO <sub>3</sub>	1.0265	1.134	72.60	35.00
0.005	Na <sub>2</sub> SO <sub>4</sub>	1.0592	1.237	73.38	36.20
0.01	None.	0.9992	1.029	72.41	34.61
1.0	"	1.0020	1.066	71.96	33.87
5.0	"		1.230	69.76	35.01
5.0	HCl	1.0249	1.473	68.38	33.54
5.0	NaI	1.0699	1.415	68.82	33.69
5.0	NaOH	1.0373	1.631	64.56	25.05
5.0	NaHCO <sub>3</sub>	1.0442	1.638	72.01	32.65
5.0	Na <sub>2</sub> SO <sub>4</sub>	1.0774	1.809	72.03	33.94
10.0	None.	1.0353	2.047	68.06	36.41

\* Determinations made with 90 per cent benzene, sp. gr. 0.8745.

† Values for dextrin, percentage concentrations 0.005 and 5.0, taken from curves. All others actual determinations.

concentrations the rate of decrease for dextrin is slightly greater than for starch. In the presence of electrolytes, however, a considerable difference is found. Most noteworthy is the very large effect of sodium hydroxide in lowering the surface tension of dextrin by almost 6 dynes in 5 per cent solution. It also has a large effect on the more concentrated starch solutions. Sodium bicar-



bonate and sulfate, while both increase the surface of dextrin, have descending curves, while for starch they ascend with increase in concentration. This would not be expected, however, in more concentrated starch emulsoids.

At the interface water-benzene, dextrin acts exactly opposite to sugar, *i.e.* there is a depression in  $\gamma$  to 1 per cent and then a steady

TABLE VI.  
*Starch.*

Concentration of starch by weight.	Electrolyte.	Specific gravity.	Relative viscosity.	Surface tension.	Surface tension at interface.*
<i>per cent</i>				<i>dynes per cm.</i>	<i>dynes per cm.</i>
0.005†	None.		1.005	72.20	34.03
0.005	HCl	1.0079	1.044	72.29	34.24
0.005	NaI	1.00525	1.016	72.73	34.86
0.005	NaOH	1.0210	1.149	72.74	35.01
0.005	NaHCO <sub>3</sub>	1.0270	1.129	72.67	34.95
0.005	Na <sub>2</sub> SO <sub>4</sub>	1.0594	1.236	73.22	35.62
0.01	None.	0.9979	1.009	72.36	34.34
0.1	"	0.9985	1.011	72.26	34.23
0.5	"		1.012	72.13	34.52
0.5	HCl	1.0089	1.088	72.23	34.28
0.5	NaI	1.0537	1.072	72.72	33.80
0.5	NaOH	1.0214	1.189	72.29	32.93
0.5	NaHCO <sub>3</sub>	1.0277	1.180	72.74	32.88
0.5	Na <sub>2</sub> SO <sub>4</sub>	1.0606	1.318	73.46	34.30
1.0	None.	1.0009	1.105	71.98	35.10
0.009‡	None.	0.9987	1.007	71.40	33.28

\* Determinations made with 90 per cent benzene, sp. gr. 0.8745.

† Values for starch, percentage concentrations 0.005 and 0.5, taken from curves. All others actual determinations.

‡ Corn-starch used. All other determinations made with soluble starch.

increase until with 10 per cent dextrin the interfacial tension is more than 2 dynes higher than in its absence. The change in state of dextrin at concentrations of 1 per cent or more undoubtedly accounts for this specific action towards benzene—a phenomenon which is not apparent at the interface solution-vapor. Exactly the same thing is true with starch except that the increase begins at only 0.1 per cent. This seems to point very clearly to a

similarity between dextrin and starch except with respect to degree of dispersion. All electrolytes in both cases produce a continuous lowering with increase in concentration with the case of sodium hydroxide again notable. This shows a marked peptization by all ions, even the  $\text{SO}_4$  ion which under ordinary circumstances has the greatest precipitating power for an emulsoid. Much of the effect upon surface tension, however, must be linked with the fact that both of these emulsifying agents are hydrous.

Dextrin and starch are not greatly different in viscosity. In both cases all electrolytes increase viscosity at small concentrations but the effect is linear while at higher concentrations solutions of the emulsoids alone increase rapidly and, as in the case of sugar, exhibit higher values than in the presence of the electrolytes. Two kinds of agglomeration must be distinguished. Agglomeration with increase in concentration into homogeneous larger particles cannot account for an increase in viscosity for, as a matter of fact, such a process should result in a *decrease* of viscosity because of the decrease in surface and the amount of bound water. Agglomeration may, however, be accomplished by increase in viscosity if there is *enclosure* of water between the masses, and not increase in the size of the particles by coalescence of smaller ones. This is what must happen, therefore, in the case of dextrin and starch, considered separately, and is an explanation of the results obtained by Meyer<sup>30</sup> on the viscosity of starch solutions.

The results on both dextrin and starch, taken separately, confirm the work of Harrison<sup>31</sup> on the viscosity formula of Hatschek<sup>32</sup> as applied to starch:  $\eta_1 = \eta (1 + Kf)$  where  $\eta_1$  is the viscosity of the emulsoid,  $\eta$  that of the pure water,  $f$  the ratio of the volume of suspended matter to the total volume, and  $K = 4.5$ . Following are results for dextrin at two concentrations:

Per cent.	$f$ (observed).	$f$ (calculated).
1	0.012	0.015
10	0.24	0.25

<sup>30</sup> Meyer, A., *Kolloidchem. Beihefte*, 1913-14, v, 1.

<sup>31</sup> Harrison, W., *J. Soc. Dyers and Colorists*, 1911, xxvii, 84.

<sup>32</sup> Hatschek, E., *Z. Chem. Ind. Kolloid*, 1910, vii, 301.

Viscosity does not therefore seem to depend upon the degree of dispersion as affected by *change in concentration* since the same formula holds true for starch; depending only on the volume of the dispersed phase. It should be noted, however, that the viscosity curve for dextrin really lies slightly above that of starch and its deviation from a linear ratio is also greater. This rather beautifully parallels the work of Oden<sup>33</sup> on sulfur sols where the viscosity of a dispersion of particles of  $0.01\ \mu$  diameter lay above that of sols of the same substance consisting of particles of  $0.1\ \mu$ . This then is the genuine case of agglomeration into *larger* homogeneous particles by coalescence setting free because of the smaller total surface some of the previously bound water and seems to point very clearly to the fact that dextrin is of the same chemical nature as starch but smaller homogeneous particles, thus confirming the view of Herzfeld and Klinger<sup>24</sup> for the first time experimentally. If, therefore, viscosity is the predominating effect upon the action of dextrin and starch as emulsifying agents, it would be expected that dextrin would be better than starch, that in dilute solutions the presence of electrolytes should be more favorable than dextrin alone, and in concentrated solutions less favorable. Such is found to be the case when Table I is examined. With viscosity, however, must be compared the interfacial tension relationships. From this point of view dextrin in the presence of sodium hydroxide which produces by adsorption of the hydroxyl ions the greatest diminution in interfacial tension should give the best emulsions, even though the viscosity is not so favorable as with dextrin alone. It is a singular fact entirely in keeping with the view here presented that 10 per cent dextrin alone with maximum viscosity produces the *heaviest cream* in every emulsion, but the *most stable* emulsion measured by time of existence is produced by 5 per cent dextrin with 0.5 M sodium hydroxide in which there is the maximum effect in reducing interfacial tension. In the case, therefore, of these emulsoids viscosity is not so important a factor as in the case of sugar. It would be interesting to discover what effect the two types of viscosity exhibited by a molecular dispersoid such as sugar and an emulsoid respectively, will have on emulsifying properties. Sugar has a true internal friction which resists some molecular motion while dextrin, starch, gelatin, albumin, etc., in solutions

<sup>33</sup> Oden, *Z. physik. Chem.*, 1912, lxxx, 709.

of equal viscosity offer no hindrance. This is related, of course, to the structure of the gel-forming substances in solution whether fibrile, net-like, or granular through the interstices of which ions or crystalloid molecules may pass unhindered, and which exhibit viscosity by virtue of deformation by the force required to drag through the capillary of a viscosimeter. It is reasonable to expect, however, that toward colloid particles the heterogeneous structure of such substances even in solution may offer resistance which is not possible towards ions or crystalloid molecules. In this case it is clear that a more effective prevention of coalescence is attained by actual film formation around each droplet than by simple hindrance of motion due to viscosity of the medium.

#### *Gum Arabic.*

Gum arabic in many ways is very similar to dextrin or starch in its relative difficulty of peptization by water, and gelatinous nature, but in general its effect at corresponding dilutions upon surface energy relationships is larger. The surface tension values are shown in Table VII. After a slight increase as observed previously there is a continuous decrease in surface tension with increase in concentration. These results are quite contrary to the findings of Quincke<sup>34</sup> and Zlobicki<sup>35</sup> who state that gum arabic increases the surface tension of water. Unlike dextrin, however, *all* of the electrolytes give lower values, with hydrochloric acid most effective in this respect as contrasted with the alkali for dextrin and starch. As a matter of fact adsorption of the hydroxyl ion causes precipitation of the colloid in 10 per cent solution. At the interface solution-benzene exactly the same effects are observed although sodium hydroxide is found to have far the greatest lowering effect in solutions up to 0.5 per cent after which the acid is most effective. In all cases the lowering is as much as 10 dynes or more from the value water-benzene in solutions from 5 to 10 per cent, a considerably greater effect than for dextrin or starch.

The viscosity shows a relation reverse to that of dextrin in that all electrolytes even in dilute solutions lower it, very markedly in

<sup>34</sup> Quincke, *Wied. Ann.*, 1888, xxxv, 582. *Drude's Ann. Phys.*, 1901, vii, series 4, 631; 1902, ix, 969; 1903, x, 507; 1904, xv, 1.

<sup>35</sup> Zlobicki, L., *Bull. Acad. Sc. Cracovie*, 1906, 488.

the more concentrated solutions. Here again in the emulsions both viscosity and interfacial tension lowering produce favorable emulsifying conditions. Thus while a 10 per cent solution with the highest viscosity produces emulsions equally good for benzene and kerosene, equally good emulsions are obtained in 0.5 per cent

TABLE VII.  
*Gum Arabic.*

Concentration of gum arabic by weight.	Electrolyte.	Specific gravity.	Relative viscosity.	Surface tension.	Surface tension at interface.*
				<i>dynes per cm.</i>	<i>dynes per cm.</i>
0.01†	None.	0.9985	1.021	72.26	34.47
0.1	"	0.9996	1.115	72.30	34.27
0.5	"		1.380	72.18	33.35
0.5	HCl	1.0087	1.114	68.75	31.40
0.5	NaI	1.0534	1.107	72.24	31.82
0.5	NaOH	1.0220	1.190	70.88	27.16
0.5	NaHCO <sub>3</sub>	1.0276	1.208	72.03	31.42
0.5	Na <sub>2</sub> SO <sub>4</sub>	1.0604	1.343	73.18	31.47
1.0	None.	1.0017	1.703	72.04	32.68
5.0	"		1.370	69.69	28.28
5.0	HCl	1.0251	2.101	61.98	20.21
5.0	NaI	1.0705	2.479	63.28	23.52
5.0‡	NaOH	1.0434	2.624	65.41	24.50
5.0	NaHCO <sub>3</sub>	1.0438	2.604	65.56	23.51
5.0	Na <sub>2</sub> SO <sub>4</sub>	1.0778	2.887	66.29	25.05
10.0	None.	1.0362	8.781	61.49	22.87

\* Determinations made with 90 per cent benzene, sp. gr. 0.8745.

† Values for gum arabic, percentage concentrations 0.5 and 5.0, taken from curves. All others actual determinations.

‡ Heavy precipitate formed. Determinations made with solution taken from top.

solution in the presence of sodium hydroxide or iodide in the case of benzene, and sodium sulfate and bicarbonate in the case of kerosene. This is another clear indication of the fact that where viscosity is chiefly operative as a favorable factor in emulsification it is largely independent of the nature and the quantity of the oil being emulsified; while on the other hand adsorption to interfaces is determined by the nature of the oil, the emulsifying agent, and the relative quantities particularly when electrolytes are present. Thus sodium hydroxide and iodide enable largest adsorp-

tion of the gum arabic at benzene-water interfaces, and sodium sulfate and bicarbonate at kerosene-water interfaces.

*Egg Albumin.*

This substance was chosen for purposes of comparison inasmuch as viscosity effects are almost negligible. Furthermore, its ten-

TABLE VIII.  
*Egg Albumin.*

Concentration of egg albumin by weight.	Electrolyte.	Specific gravity.	Relative viscosity.	Surface tension.	Surface tension at interface.*
				<i>dynes per cm.</i>	<i>dynes per cm.</i>
0.01†	None.	0.9980	1.005	72.28	33.96
0.05	"		1.014	70.30	28.72
0.05‡	HCl	1.0078	1.060	65.63	23.58
0.05	NaI	1.0532	1.022	66.88	23.02
0.05	NaOH	1.0206	1.118	57.51	15.96
0.05	NaHCO <sub>3</sub>	1.0262	1.124	66.43	25.11
0.05	Na <sub>2</sub> SO <sub>4</sub>	1.0595	1.248	61.35	24.81
0.1	None.	0.9996	1.024	68.99	26.38
0.409	"		1.054	66.27	20.39
0.409	HCl	1.0075	1.373	62.82	20.24
0.409	NaI	1.0537	1.057	59.64	19.66
0.409	NaOH	1.0214	1.169	52.62	12.03
0.409	NaHCO <sub>3</sub>	1.0276	1.147	58.20	20.58
0.409	Na <sub>2</sub> SO <sub>4</sub>	1.0610	1.274	60.76	21.20
0.415	None.	0.9994	1.056	66.30	20.15
0.415§	"	1.0000	1.056	65.09	25.49
0.918	"	1.0012	1.057	61.67	21.52

\* Determinations made with 90 per cent benzene, sp. gr. 0.8745.

† Values for egg albumin, percentage concentrations 0.05 and 0.409, taken from curves. All others actual determinations.

‡ Heavy precipitate formed in both cases with HCl. Determinations made with solution taken from top.

§ Determinations made with fresh solution. All other determinations with egg albumin were made with solutions 2 weeks old. In the latter case albumin settled out of solution which was filtered off without redetermining the percentage strength of the solution.

dency to form films by adsorption at liquid interfaces may be easily seen with the eye if water is dropped into benzene and not shaken. The surface tension of egg albumin solutions has been the subject of considerable controversy. Quincke<sup>34</sup> and Zlobicki<sup>35</sup> and others state that it diminishes the tension at water-vapor surfaces,

while Iscovesco<sup>36</sup> found that it increases the surface tension. As a matter of fact it does both—increasing in the most dilute solutions and decreasing in the more concentrated. The surface tension values shown in Table VIII are reminiscent of gum arabic in that all electrolytes lower, with sodium hydroxide most effective. Hydrochloric acid, of course, is adsorbed and causes precipitation. The same relations hold true at the solution-benzene interface. Thus 0.5 M sodium hydroxide in 0.918 per cent egg albumin lowers the interfacial tension by more than 22 dynes, considerably the greatest effect observed in any of the emulsifying agents. Chemical action such as the formation of sodium albuminate and hydrolytic cleavage account for this.<sup>37</sup>

The viscosity of albumin emulsoids has been the subject of numerous investigations.<sup>38</sup> Hardy<sup>39</sup> and Bottazzi<sup>40</sup> have shown that viscosity is dependent upon protein ions, is at a minimum when these are absent, and that addition of acids and bases should increase viscosity. It is clearly evident from Table VIII that in these experiments variations in viscosity are not important. Efficiency as an emulsifying agent must, therefore, be related here to adsorption and film formation and it would be expected that the best results would be obtained in the presence of sodium hydroxide which lowers the interfacial tension to the greatest extent. This is found to be true. Excellent emulsions were obtained with every solution of egg albumin, all by far better than any with the preceding four emulsifying agents, of these the most stable had present sodium hydroxide. This is entirely in keeping with the finding of Zsigmondy<sup>26</sup> who gives the following gold numbers for proteins contained in egg albumin:

	Gold number.
Globulin.....	0.02 —0.05
Ovomucoid.....	0.04 —0.08
Crystallized egg albumin.....	2.0 —8.0
Other con-albumin.....	0.03 —0.05
Alkali albuminate.....	0.006—0.04

<sup>36</sup> Iscovesco, H., *Compt. rend. Soc. biol.*, 1910, lxix, 622; 1911, lxx, 11, 66.

<sup>37</sup> Handovsky, H., *Biochem. Z.*, 1910, xxv, 510.

<sup>38</sup> Cf. Ostwald,<sup>26</sup> p. 156.

<sup>39</sup> Hardy, W. B., *J. Physiol.*, 1905-06, xxxiii, 251.

<sup>40</sup> Bottazzi, F., *Atti. R. Accad. Lincei*, 1913, xxii, 141, 263.

## SUMMARY.

1. The purpose of the researches described in this paper has been to determine by quantitative measurement what the attributes essential for an emulsifying agent or colloid protector are, and what kind of substance is best in this respect among non-electrolytes.

2. Quantitative measurements are made by the most accurate methods known of surface tension, interfacial tension, and viscosity of solutions of sugars, dextrin, starch, gum arabic, and egg albumin at all concentrations, both alone and in the presence of each of five electrolytes: hydrochloric acid, sodium hydroxide, iodide sulfate, and bicarbonate. To enable a practical test of theoretical principles a study of actual emulsions is also made with each of the above five substances alone and in the presence of each of the five electrolytes.

3. There are two factors of predominating importance in considering sugar as an emulsifying agent, *i.e.* viscosity and ability to lower the interfacial tension, with viscosity of primary importance. The chemical nature of the oil used in making emulsions will determine to some extent the efficiency of sugar as an emulsifying agent while the chemical nature of the liquids being emulsified is also important.

4. The inversion of sucrose, measured for the first time by surface tension methods, is accompanied by a slight increase in surface tension. Experimental data shown herewith indicate that invert sugar is no more efficient than is sucrose in holding up suspensoids or emulsoids.

5. For the first time experimental confirmation is given of the fact that dextrin is of the same chemical nature as starch but smaller homogeneous particles.

6. Dextrin is found to be a better emulsifying agent than starch. Viscosity and lowering of interfacial tension is here again of predominating importance in producing the best emulsions, with, however, viscosity of only secondary importance.

7. Two types of viscosity from the standpoint of emulsification are discussed: that of a molecular dispersoid which has a true internal friction resisting some molecular motion and that of an emulsoid which offers no hindrance.



8. Lowering of interfacial tension and viscosity produce favorable emulsifying conditions for solutions of gum arabic.

9. Efficiency as an emulsifying agent for egg albumin is related to adsorption of film formation. Better emulsions were obtained with egg albumin than with any one of the four other substances under consideration.

10. No one general rule can be made as to the effect which produces the best emulsions for any one substance nor can any one generalization be made for the effect which produces best emulsions for all substances under all conditions. Those which seem to be of predominating importance are viscosity and film formation, the latter effect being of primary importance since it was with egg albumin, where a film formation is quite marked, that the best emulsions were obtained among all five of the substances under consideration in this study.

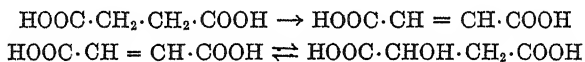
## THE ACTION OF MUSCLE TISSUE ON FUMARIC, MALEIC, GLUTACONIC, AND MALIC ACIDS.

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The most recent experiments of Einbeck (1) on the oxidation of succinic acid by muscle extract lead him to conclude that the reaction proceeds primarily with the formation of fumaric acid and that the latter acid, by means of a balanced reaction, is converted into optically *inactive* malic acid to the extent of about 75 per cent:



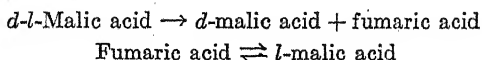
Passing reference may be made to the fact that Battelli and Stern (2) had already indicated the formation of optically inactive malic acid from succinic acid by means of their "succinicoxydon" though their chemical identification of the acid can hardly be regarded as convincing. Subsequently Einbeck (3) regarded fumaric acid as the sole product of this reaction; an error which he has himself corrected.

The formation of optically inactive malic acid as the result of enzyme action seemed so anomalous to the writer that it was decided to repeat Einbeck's experiments, especially as Einbeck gives no details of the experimental conditions under which the polarimetric observations were made. The result of the writer's experiments is to show that exclusively *l*-malic acid results from the action of muscle enzymes on salts of fumaric acid. This asymmetric synthesis possesses additional interest from the fact of the stereochemical similarity of *l*-malic acid and *d*-lactic acid and may be of significance in connection with the hypothetical formation of malic and lactic acids in the intermediary catabolism of glutamic,  $\beta$ -hydroxyglutamic, and allied amino-acids (4).

Experiments with maleic acid showed that its behavior was entirely different from the stereoisomeric fumaric acid. No trace of optically active malic acid was formed on digestion of its sodium salt with muscle and the substance was practically unchanged. This result accords with Thunberg's (5) observation of the deleterious affect of maleic acid on the respiratory exchange of muscle, in marked contrast with the effect of fumaric acid.

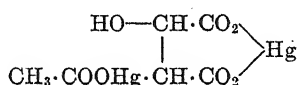
Glutaconic acid, the homologue of fumaric acid and presumably possessing a "fumaroid" rather than "maleinoid" structure, gave a small amount of  $\beta$ -hydroxyglutaric acid which was of necessity optically inactive owing to its symmetrical constitution. The bulk of the glutaconic acid was recovered unchanged however. It is perhaps unfortunate that the action of the muscle enzymes on glutaconic acid should not have given a more definite result, since, as is well known, there is still a certain amount of doubt as to the actual structure of glutaconic acid in solution. Its undoubtedly mobile hydrogen atom has been variously represented by different formulas, and there was a possibility that the action of enzymes might have thrown some light on the problem. At any rate, it is clear that glutaconic acid undergoes a change with muscle enzymes to an almost insignificant extent when compared with fumaric acid.

Finally, experiments were made in which the sodium salt of inactive malic acid was subjected to the action of muscle enzymes. The specific rotation of the residual malic acid was found to become progressively more and more *dextro-rotatory* reaching an equilibrium in about 6 hours and then remaining practically constant or receding slightly. The explanation of this result is ascribed to the preferential conversion of the levo component of inactive malic acid into fumaric acid. Had the dextro and levo components been convertible into fumaric acid with equal ease the whole of the residual malic acid would have been the levo variety since this active acid is in equilibrium with fumaric acid as already described. The observed formation of *d*-malic acid may be represented as follows:



It would appear from the results that *d*-malic acid is not readily converted into fumaric acid by the action of muscle enzymes.

The asymmetric production of *l*-malic acid from fumaric acid by the action of muscle enzymes has hitherto not been simulated by ordinary chemical reagents of known structure. Reference may be made to an unsuccessful attempt at such an asymmetric synthesis of malic acid from maleic acid. Biilmann (6) has shown that maleic acid gives a complex mercury salt on reacting with mercuric acetate which on decomposition gives malic acid almost quantitatively. Biilmann's salt has the following structure containing an asymmetric carbon atom:



It was thought possible that by using the mercury salt of lactic acid in place of the acetate that optically active malic acid might result. The *l*-lactate gave the desired salt, but the malic acid resulting from its decomposition was entirely optically inactive.

#### EXPERIMENTAL.

*Fumaric Acid*.—In each experiment 5 gm. of pure fumaric acid were converted into the neutral sodium salt and diluted to 500 cc. This solution was mixed with 100 gm. of minced sterile muscle from either a rabbit or dog. Polarimetric observations showed that equilibrium was reached between fumaric and malic acids in about 6 hours so that antiseptics were unnecessary unless digestion was prolonged.

At the end of 6 hours digestion at 40°, polarimetric observation on the filtrate obtained by coagulating a portion of the fluid and adding solid uranium acetate showed that from 3 to 3.5 gm. of *l*-malic acid had been formed. The *l*-malic acid was isolated as follows: The digestion mixture which was distinctly acid was coagulated by heating on a steam bath and the coagulated proteins were filtered off and washed. The filtrate was then concentrated under reduced pressure to a volume of about 200 cc. A slight excess of lead acetate (20 gm.) was then added in concentrated solution and then ammonia until the supernatant fluid was no longer acid. A third of the volume of alcohol was then added and the precipitate, containing the fumarate and malate, was allowed to stand over night in a cold place. The precipitate was

then filtered off, washed with cold water, and decomposed with a slight excess of sulfuric acid. The filtrate was then made alkaline by the addition of crystallized barium hydroxide (18 gm.) and the precipitate, containing fumaric and phosphoric acids, was removed by filtration. The malic acid in the filtrate was then recovered by precipitation in neutral solution as the silver salt in the customary fashion. The precipitate on decomposition with hydrogen sulfide gave 2.67 gm. of crude malic acid which slowly crystallized from its concentrated aqueous solution. Its final purification was readily effected by dissolving it in water (15 cc.) and extracting it with butyl alcohol under reduced pressure in the apparatus previously described (7). On evaporating the butyl alcohol and dissolving the residue in a minimum of water, 2.4 gm. of pure crystalline malic acid were readily obtained.

*Analysis.* 0.1474 gm. substance: 0.2102 gm.  $\text{CO}_2$ , 0.0591 gm.  $\text{H}_2\text{O}$ .

$\text{C}_4\text{H}_6\text{O}_5$ . Calculated. C 35.8, H 4.48.

Found. " 35.7 " 4.46.

*Optical Rotation.* In water.  $c = 16.6$ ;  $l = 2.2$ ;  $\alpha = -0.62^\circ$

$[\alpha]_D^{20} = -1.7^\circ$

In pyridine.  $c = 5.0$ ;  $l = 2.2$ ;  $\alpha = -3.30^\circ$

$[\alpha]_D^{20} = -30.0^\circ$

In 10 per cent uranium acetate.  $c = 0.5$ ;  $l = 2.2$ ;

$\alpha = -5.30^\circ$

$[\alpha]_D^{20} = -482^\circ$

The above figures prove conclusively that muscle enzymes acting on fumaric acid convert a large proportion of the latter substance into optically pure *l*-malic acid.

*Maleic Acid.*—Freshly distilled maleic anhydride (5 gm.) was converted into the neutral sodium salt and then digested in 1 per cent solution with fresh muscle (100 gm.). No optically active malic acid could be detected on adding uranium acetate and examining the filtrate in the polarimeter, while much unchanged maleic acid was left in the solution and could be recovered on extraction with ether. It was noted that 1 per cent solutions of sodium maleate exercised a marked antiseptic action on muscle extract. The experiments with maleic acid were essentially similar to those described for fumaric acid but gave uniformly negative results.

*Glutaconic Acid.*—This acid was prepared in the usual way by the hydrolysis with hydrochloric acid of the ethyl ester of glutaconic dicarboxylic acid. 5 gm. of the pure acid were neutralized with soda and the solution (500 cc.) digested for 20 hours at 40° with 100 gm. of fresh dog muscle. The solution was then heated on the steam bath to coagulate proteins and filtered. The filtrate had a trifling rotation in a 2.2 dm. tube which was only increased to 0.10° on adding uranium acetate. The bulk of the filtrate was concentrated to 100 cc., strongly acidified with phosphoric acid, and then extracted with ether in a continuous extractor for 12 hours. The ether extract on treatment with a little water deposited much unchanged glutaconic acid. The mother liquor from the crystals was well dried *in vacuo* and then treated in the cold with excess of acetyl chloride. A solid mass at once separated which also contained much glutaconic acid. It was treated with warm chloroform in which the latter acid is insoluble and filtered off. In all slightly over 3 gm. of unchanged glutaconic acid were recovered. On concentrating the chloroform mother liquor a small amount (0.5 gm.) of crystals which after recrystallization from chloroform melted at 87°, was obtained and proved to be  $\beta$ -acetoxyglutaric anhydride. The substance was identical in properties with those given by Blaise (8) who also obtained it by the action of acetyl chloride on  $\beta$ -hydroxyglutaric acid. The results of the experiment indicate that muscle enzymes act much less vigorously on glutaconic than on fumaric acid but that a small amount of symmetrical  $\beta$ -hydroxyglutaric acid is produced.

*Inactive Malic Acid.*—The acid was recrystallized by dissolving 10 gm. in 10 cc. of absolute alcohol and adding 30 cc. of chloroform. Good crystals of the inactive acid (7 gm.) were readily obtained by this method. 5 gm. of the acid were converted into the neutral sodium salt and the solution diluted to 500 cc. was digested with 110 gm. of rabbit muscle at 40°. After varying lengths of time portions of the mixture (20 cc.) were withdrawn, heated in a water bath to coagulate proteins, and then shaken with 2.5 gm. of uranium acetate. The filtrate was then polarized in a 2.2 dm. tube. The results were as follows:

Time.	Rotation.	Approximate amount of <i>d</i> -malic acid.
<i>hrs.</i>		<i>gm.</i>
	+0.02	0.13
0.5	+0.31	0.27
1.5	+0.60	0.53
4.5	+1.17	0.52
7.5	+1.15	
23.0	+1.10	0.50

On working up the digestion mixture as described for the fumaric acid experiments, it was found that the residual malic acid recovered was dextro-rotatory as the preliminary polarimetric observations indicated. Small amounts of fumaric acid were also separated. It is therefore concluded that the levo component of inactive malic acid passes over into fumaric acid more readily than the dextro component.

In conclusion reference may be made to the bis-phenylhydrazide of inactive malic acid which seems not to have been previously prepared, and which might prove useful for identification purposes. It was obtained by heating inactive malic acid (1 mol) in 10 per cent aqueous solution with phenylhydrazine (3 mols) and acetic acid (1.5 mols). After 3 or 4 hours heating on the water bath the hydrazide separated out of solution as a granular crystalline mass, which was filtered off and washed with a little alcohol. The substance is much less soluble in hot alcohol than the corresponding derivative of active malic acid described by Bülow (9) and by Fischer and Passmore (10) and is best crystallized by dissolving it in a little hot glacial acetic acid and adding an equal volume of water. It crystallizes readily in colorless prisms melting at 221–224° (uncorrected).

<i>Analysis.</i>	0.1404 gm. substance:	0.3136 gm. CO <sub>2</sub> ,	0.0731 gm. H <sub>2</sub> O.
	C <sub>18</sub> H <sub>18</sub> N <sub>4</sub> O <sub>3</sub> .	Calculated.	C 61.1, H 5.73.
		Found.	" 60.9 " 5.79.

#### SUMMARY.

The enzymes of muscle tissue convert salts of fumaric acid into optically active malic acid. The levo variety is exclusively formed as opposed to Einbeck's statement that inactive malic acid is produced.

Maleic acid under similar conditions gives no optically active malic acid while glutaconic acid gives a little symmetrical  $\beta$ -hydroxyglutaric acid. On subjecting inactive malic acid to the action of muscle enzymes, the residual malic acid contains an excess of the dextro component while some fumaric acid is produced. It would appear that the levo component is more readily converted into fumaric acid than the dextro component.

The bis-phenylhydrazide of inactive malic acid is described.

An attempt to effect an asymmetric synthesis of active malic acid by the action of mercury *l*-lactate on maleic acid was unsuccessful.

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## DETERMINATION OF FATTY ACIDS (AND CHOLESTEROL) IN SMALL AMOUNTS OF BLOOD PLASMA.

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Fatty acids being the essential metabolic constituents of the lipoids of the blood, their determination along with that of cholesterol gives the best measure of its gross lipid content. When time and material are available these substances are probably best determined by the gravimetric method (1), by which they are isolated and weighed; but the gravimetric procedure is tedious and the amounts of blood necessary are not readily obtainable from small experimental animals, and even in the case of human beings it is often not desirable to remove the amount necessary for duplicate determinations, especially on plasma, since at least 100 cc. of blood would be required. In small animals the removal of such a large amount of blood (2) may set up reactions (e.g. lipemia), which would entirely destroy the value of any subsequent determinations. For these reasons a method was devised some years ago which required only small amounts of blood (3) and by which determinations could be made in a relatively short time. The method depended on the extraction of the lipid material from the blood by alcohol-ether, saponification of the lipid by alkali, and determination of the fatty acid and cholesterol together, by the use of the nephelometer, the suspensions on which the measurement depends being formed in water by the use of an acid. The suspension is not stable but changes in nephelometric value rather rapidly, so that conditions established for precipitation and reading must be closely adhered to. Cholesterol was determined separately in another sample of extract by the colorimetric method (4), the value so obtained subtracted from the value for "total fat" obtained above and the difference taken as the fatty acid value. Figures for fatty acid obtained

by this method are higher than those obtained by the gravimetric methods, one reason for which, as pointed out by Csonka (5), is the fact that the nephelometric value of cholesterol is considerably greater than that of the fatty acids. Csonka observed further that the nephelometric values for various fatty acids as obtained by the acid precipitation were not equal and that, therefore, the use of the method might lead to quite incorrect results. For these reasons a revision of the method was called for and the present paper records the results of attempts made to obviate the difficulties mentioned.

The alcohol-ether treatment has been found to give a sufficiently close extraction of the blood lipoids, a finding which has been borne out by the work of other investigators (6, 7), and it has therefore been retained.

To neutralize the error due to cholesterol a mixed standard of fatty acid and cholesterol approximating the proportions found in the blood was first tried but was given up, since although in normal blood the relation between fatty acid and cholesterol is fairly constant such is not the case in blood under abnormal conditions. Also, the relation between fatty acid and cholesterol in blood plasma was found to be different in different animals and a separate standard would be necessary for each.

The use of a table of corrections constructed from values obtained with known mixtures of fatty acid and cholesterol gave more promise of success since although cholesterol and fatty acids do not always vary to the same extent, their variations are generally in the same direction and a fair approximation to the true value may be obtained. But both schemes are rendered unsafe by uncertainty regarding true cholesterol values in blood since it has been made very probable by work in this and other (8, 9) laboratories that there is present in blood another substance than cholesterol which gives at least the color reactions of cholesterol and may behave like it in other ways.

It was felt therefore that if the nephelometric determination were to be retained two things should be accomplished: (a) a separation of the cholesterol from the fatty acid; and (b) a method of precipitation for the fatty acids which would give values as nearly alike as possible for the different fatty acids and especially for oleic and palmitic acids, which as far as the limited data on the

subject show, are the commonest ones found in blood; or failing in that to find a method of precipitation which would give values close to those found for the blood fatty acids by the gravimetric method. In the procedure finally adopted the cholesterol is separated from the fatty acid by taking advantage of the fact that dry soaps are practically insoluble in cold chloroform while cholesterol dissolves readily.

Two methods for the nephelometric determination of the fatty acids were attempted, one of which (the acid precipitation) gives results which are in good agreement with the values obtained gravimetrically and which may, therefore, for the present be recommended for use in the determination of fatty acids in blood, although it fails with certain of the known fatty acids and their mixtures; and another (calcium precipitation) which gives results on blood considerably below those obtained gravimetrically, but which with the known higher fatty acids and their mixtures gives results which are on the whole considerably more accurate than those obtained by the acid precipitation.

In Table I are contained the nephelometric values of different mixtures of oleic and palmitic acids using the two methods of precipitation under different conditions, and with different standards. Oleic and palmitic acids were dissolved in alcohol so that each 5 cc. contained 2 mg. of the acids or mixtures of them made in different proportions. Determination of the contained fatty acid was made as follows.

*Acid Precipitation.*—5 cc. of the fatty acid mixture were run with stirring into 100 cc. of distilled water in a beaker, then 10 cc. of a mixture of 1 part of concentrated hydrochloric acid and 3 parts of water added with stirring. After standing for 3 to 5 minutes comparison was made in the nephelometer with the standard similarly prepared.

*Calcium Precipitation.*—100 cc. of distilled water, free from carbon dioxide, were measured into a beaker and 4 cc. of the calcium reagent (10 per cent calcium acetate to which has been added enough sodium hydroxide solution to produce a permanent precipitate which was then filtered off) added. 5 cc. of the fatty acid solution were run in from a pipette with stirring, and readings made by comparison with the standard similarly prepared.

The values are expressed in terms of the standard taken as 100 and each is the average of two closely agreeing determinations.

An examination of the first line of the table—cold acid precipitation with oleic acid as standard—shows the great variation in nephelometric values which may be obtained with different mixtures of the two acids. Further examination brings out the important fact that there is a sharp break in the values between oleic 60 palmitic 40, and oleic 50 palmitic 50, those from the

TABLE I.  
*Nephelometric Values of Mixtures of Oleic and Palmitic Acids (Standard = 100).*

Standard.	Oleic 90 per cent, Palmitic 10 per cent.	Oleic 80 per cent, Palmitic 20 per cent.	Oleic 70 per cent, Palmitic 30 per cent.	Oleic 60 per cent, Palmitic 40 per cent.	Oleic 50 per cent, Palmitic 50 per cent.	Oleic 40 per cent, Palmitic 60 per cent.	Oleic 30 per cent, Palmitic 70 per cent.	Oleic 20 per cent, Palmitic 80 per cent.	Oleic 10 per cent, Palmitic 90 per cent.	Palmitic 100 per cent.
Cold acid precipitation (20°C.).										
Oleic 100 per cent.....	103	97	96	97	176	200	210	230	250	176
Hot acid precipitation (50-60°C.).										
Oleic 100 per cent.....	100	90	93	79	81	79	77	70	79	66
Oleic 60 per cent, palmitic 40 per cent.....	107	110	107	100	104	100	93	100	100	97
Calcium precipitation.										
Oleic 50 per cent, pal- mitic 50 per cent.....	103	101	100	97		91	90	88	88	87
Oleic 100 per cent.....	100	97	94	96	94	87	83	85	88	81

former point onward being very much higher than the preceding ones, which are quite constant and practically identical with the standard. In seeking an explanation for the sharp break in values it was recalled that oleic acid in suspension would be liquid (forming an "emulsion colloid"), palmitic acid in suspension would be solid (forming a "suspension colloid"), while mixtures of the two would be either liquid or solid depending on the proportion of each. Evidently in water at room temperature the mixtures containing 60 per cent or more of oleic acid are liquid

and behave like oleic acid while those containing less than 60 per cent are solid and behave like palmitic acid. That, however, there are other factors involved, is rendered probable by the fact that certain mixtures of oleic and palmitic acids give higher values than either palmitic or oleic acid, recalling the similar behavior of the melting points of mixtures of the fatty acids. From the results it might be inferred that any mixture of fatty acids of a lower melting (or solidifying) point than oleic 60 palmitic 40 may be determined by the acid precipitation method with a good degree of accuracy; an inference which is borne out by the fact shown in Table II, that the acid precipitation method is accurate for the blood fatty acids, the melting and solidifying points of which are always below those of this oleic-palmitic acid mixture.

Thinking to overcome the effect of these differences in state, determinations were carried out at a temperature at which all the fatty acids would be liquid. The results of this experiment may be seen from the second and third lines of Table I—the nephelometric values are reversed, palmitic acid and its mixtures with oleic acid giving lower values than oleic acid alone. By changing the standard from oleic acid alone to a mixture of 60 per cent oleic and 40 per cent palmitic acids, values are obtained for the different mixtures sufficiently close to each other (Line 3, Table I) to warrant the belief that satisfactory determinations on blood fatty acids could be made in this way. Nevertheless, the use of hot solutions is inconvenient and might lead to inaccuracies, since in the small nephelometer tubes rapid cooling takes place and unless readings are made promptly erroneous values are obtained. Moreover, the influence of temperature on the solubility of the fatty acids and their mixtures is an unknown quantity.

For these reasons it was decided to attempt some other method of precipitation and consideration was given to the metals, most of which give insoluble salts with the fatty acids. It was found that precipitation by metals in acid solution gave the same results as precipitation by acid alone and that, therefore, an alkaline reagent must be used. The necessity of precipitation in alkaline solution eliminates a good many of the metals and the presence of traces of substances such as chlorides in the blood digests eliminates others, so that the choice of precipitating metal was pretty well limited to those of the calcium group and the final

choice was calcium. Examination of Lines 4 and 5 of Table I (calcium precipitation) shows that with oleic acid as standard the nephelometric values of mixtures of oleic and palmitic acids fall off gradually until with palmitic acid alone the difference is 19 per cent. Using a mixture of equal parts of oleic and palmitic acids as standard, satisfactory values are obtained over a considerable range of mixtures and the value for palmitic acid is only 13 per cent below that of the standard. Calcium would therefore seem to be a satisfactory precipitant for the fatty acids of blood especially as the range of fatty acid mixture is probably not great—in fact the blood fatty acid mixture ordinarily has a melting point below that of a mixture of 60 parts oleic and 40 parts palmitic acid (see Column 6, Table II). Determination of the nephelometric values of the other higher fatty acids by the calcium precipitation gives the following values using a 50 per cent mixture of oleic and palmitic acids as the standard—a sufficiently close agreement considering the fact that these acids do not occur singly in the blood but always as mixtures.

	<i>per cent</i>
Oleic.....	100
Stearic.....	83
Palmitic.....	86
Linoleic.....	94
Lauric.....	94

The lower fatty acids, capric, caprylic, and lower, do not give precipitates with either calcium or acid in these dilutions.

The precipitation of the fatty acids with calcium gives a cloud which is excellent for nephelometric purposes, being easily read, of practically the same tint for all the fatty acids and stable—the same values being obtained whether the reading is made at once or at any time up to 1 hour.

The promise thus held out for the usefulness of the calcium precipitation in the determination of blood fatty acids is not, however, fulfilled when the method is applied to blood, the values obtained being much below those obtained by the gravimetric method which is used as the basic method (Table II, Columns 1 and 3). Its failure is not easy to explain. As noted above it gives good results with known fatty acids and with various mixtures of the two commoner ones (oleic and palmitic acids) either

when used directly with the fatty acids or after these have been submitted to the treatment with alkali, drying, and separation with cold chloroform, etc., as in the method for blood described below. To get some information as to the factors responsible for the abnormal calcium values the following experiments were made: (a) The fatty acids obtained in the gravimetric determinations were dissolved in alcohol and made up to a convenient volume (50 or 100 cc.) and the fatty acid content was measured nephelometrically by means of the acid precipitation and the calcium precipitation. The results are given in Columns 4 and 5 of Table II. The values obtained by the acid precipitation (Column 4) agree quite well with the theoretical values as shown by the gravimetric determinations (Column 1) and also with the values obtained by the acid precipitation directly on the blood (Column 2). The values obtained by the calcium precipitation (Column 5) are irregular and in general much lower than the theoretical values. Sometimes they agree with the values obtained by the calcium precipitation directly on blood (Column 3) but mostly they do not. (b) Samples of the mixed fatty acids of beef blood prepared in large amounts for another purpose were submitted to the Twitchell (10) lead salt method for separating the solid and liquid fatty acids. Portions of the fractions thus obtained were dissolved in alcohol and measured by the two methods of precipitation as above. It was found that while theoretical values were obtained by both methods of precipitation with the solid fraction, only the acid precipitation gave good results with the liquid fraction, the calcium results being much too low.

These experiments indicate that there is present in the blood fatty acid mixture, probably in the liquid fraction, some material which precipitates with acid but not with calcium. Whether the "unknown" substance is fatty acid in a form not precipitable by calcium—anhydride, lactone, etc.—but which should properly be considered as fatty acid in the determination, or whether it is some other type of substance must be left for further work to decide but for the present it is assumed that the former is the case and that, therefore, the calcium precipitation cannot be recommended for the determination of the fatty acids of blood.

Since the calcium precipitation is not available for the fatty acids of blood, attention was again directed to the acid precipita-



tion in the hope of finding conditions in which it might be used. A hint was obtained from Table I. As noted above, in Line 1 it may be seen that using the cold acid precipitation with oleic acid as standard, values within 5 per cent of the true value may be obtained with mixtures of oleic and palmitic acids from 90 to 60 per cent oleic acid but not with mixtures containing less oleic acid. Determination of melting and solidifying points of the 60-40 mixture gave values of 48-49°C. and 41-43°C., respectively, both somewhat above the average body temperature of warm blooded animals. Having previously found in a few samples that the solidifying point of the blood fatty acid mixture was generally below body temperature, determinations were made of melting and solidifying points of a number of samples of blood fatty acids from different animals, the results of which are given in Columns 6 and 7 of Table II. From these it may be seen that both melting and solidifying points are very close to body temperature, the former being generally above 37°C. and the latter below it. The blood fatty acid mixtures then apparently fall within the limits shown in Table I, Line 1, where good results are obtainable by the acid precipitation, *i.e.* they melt at a point below the melting point of the 60 per cent oleic-40 per cent palmitic acid mixture and that, therefore, the acid precipitation might probably be used for determinations of the fatty acids of blood. The series of comparative determinations (Columns 1 and 2, Table II) using the Kumagawa-Suto procedure as standard show this to be the case, the values obtained by the acid precipitation generally falling within 5 per cent of the values obtained by the gravimetric method. The acid precipitation method is, of course, open to the objection that if fatty acid mixtures occurred in blood which were of higher melting point than the 60-40 mixture noted above values obtained by the acid precipitation would be inaccurate. But such does not appear to be the case, probably for the reason that the transport of fatty acids or their compounds which were solid at body temperatures would be difficult or impossible.

Fatty acids added to the alcohol-ether extract of blood are recoverable by this method within 5 per cent as are also fatty acids from a fatty acid-cholesterol mixture submitted to separation and determination as in the method. In spite of the theoretical limitations to its use mentioned above, the acid precipitation

method has, therefore, been adopted for determination of the fatty acids. Its absolute accuracy is probably not far from 5 per cent of the true value since the agreement of values obtained by it and by the gravimetric method is generally within that figure.

TABLE II.  
*Fatty Acids of Blood Plasma.*

Sample.	Date.	Fatty acids per 100 cc. of blood.			Values of blood fatty acid (Column 1).		Melting point.	Solidifying point.
		Gravimetric. (1)	Micro (acid). (2)	Micro (Ca). (3)	Micro (acid). (4)	Micro (Ca). (5)		
	1921	mg.	mg.	mg.	mg.	mg.	°C.	°C.
Beef.....	Nov. 7	160		150				
" .....	" 9	170		117	164	107		
" .....	" 14	98		75	100	72		
" .....	" 18	172	175	133	160	60		
" .....	" 20	184		100	172	120		
Dog.....	" 24	400	428	300	390	250		
Beef.....	" 28	154		100	140	92	40	35
" .....	" 29	160		136	136	80		
Dog.....	Dec. 3	300	279	177	296	120	42	38
" .....	" 7	256	237	136	270	168	38	34
" .....	" 14	188	194	125	214	130	37	31
" .....	" 19	136	146	77	132	80	38	33
" .....	" 20	180	187	150	181	140	42	36
Pig.....	" 23	160	170	155	150	94	38	32
" .....	" 29	204	190	150	210	154	37	32
" .....	" 30	166	175	136	170	100	40	35
" .....	1922							
" .....	Jan. 13	330	210	136			43	38
Beef.....	" 13	192	193				39	36
" .....	" 14	188	193				40	36
A mixture of 60 per cent oleic and 40 per cent palmitic.							48-49	41-43

For the separation of the fatty acids from cholesterol in the saponification residue, cold chloroform is used, which dissolves the cholesterol readily but the soaps very slightly. Large numbers of separations on known mixtures of fatty acids and cholesterol made as directed below show that the separation is accurate to within 5 per cent of the true value for either cholesterol or fatty acid. Either cholesterol or fatty acid added to blood extracts is

recoverable within this figure. Values for cholesterol in blood obtained by this method, as by the similar method previously described (11), are always lower than those made on the alcohol-ether blood extract without saponification but are in good agreement with those obtained by other saponification methods. Similar differences between values obtained on blood extracts with and without saponification have been reported by other investigators (8, 9) and since ordinary cholesterol is not affected by the saponification it seems probable that some substance is present in blood which gives the cholesterol color reaction but which is sensitive to alkali. According to the investigators mentioned above the unknown substance is ordinarily present in considerable amounts (50 to 100 mg. per 100 cc. of blood), occurs in large amount in biliary disturbances (9) and may be entirely absent in cancerous conditions (8).

Briefly, the results of the present investigation are as follows: The lipid substances may be satisfactorily extracted from blood plasma by treatment with excess of hot alcohol-ether. In these extracts the fatty acid constituents of the lipoids may be separated from the cholesterol by saponification of the extract and extraction of the saponification residue with cold chloroform, which extracts the cholesterol, leaving the fatty acids (soaps) behind. These may be extracted from the residue by hot alcohol. Determination of the cholesterol is made on the chloroform extract after suitable concentration, by the Liebermann-Burchard reaction.

Determination of fatty acid is made on the alcohol extract nephelometrically using the acid precipitation method. The procedure is as follows.

#### *Procedure.*

*Extraction and Saponification.*—5 cc. of blood plasma are measured into a 100 cc. flask containing about 75 cc. of a mixture of 3 parts alcohol and 1 part ether (both redistilled). The plasma is made to enter in a slow stream of drops and the liquid in the flask is kept rotating rapidly to prevent the formation of large aggregates of precipitate. At once, or after standing till a convenient time, the flask is immersed in boiling water with frequent and strong rotation (to prevent superheating) until the liquid begins to boil, then cooled to room temperature, made up to

volume, mixed, and filtered. For the determination a volume (10 to 20 cc.), containing about 2 mg. of fatty acid, is measured into a small Erlenmeyer flask (50 to 100 cc.) of Non-sol glass (Pyrex is less suitable because it is readily attacked by the strong alkali), 0.1 cc. of concentrated NaOH made from sodium is added and the mixture evaporated on the water bath. When the volume of liquid has been reduced to a few drops the flask should be rotated or shaken occasionally so as to distribute the liquid evenly over the bottom (but not over the sides). The drying is then continued until only 2 or 3 drops of liquid remain and the odor of alcohol is entirely gone. The alkali is then *partially* neutralized by the addition of 0.1 cc. of dilute sulfuric acid (1 volume concentrated acid, 3 volumes water), and the liquid well mixed and distributed over the bottom of the flask as before. The drying is then continued on the water bath until the residue is dry and all the moisture has disappeared from the sides of the flask. The process of drying is a very important step in the method, since the separation is not quantitative if the drying is either carried too far (in which case some of the cholesterol cannot be recovered by the cold treatment), or not far enough (when a part of the soap or fatty acids is extracted with the cholesterol). The amount of acid added should be somewhat less than enough to neutralize the alkali since otherwise fatty acids would be set free and dissolve in the chloroform. For the same reason the added acid should be well mixed with the residue in the flask so as to insure its complete neutralization. If there is not enough liquid in the flask to allow complete mixing, a drop or two of distilled water should be added. The reason for the addition of acid is twofold, first to prevent destruction of cholesterol by the strong alkali (for contrary to the statements in the literature we have found that cholesterol is altered—at least as far as its color producing properties are concerned—by heating with strong alkali), and second by the formation of the crystalline sodium sulfate the residue is made porous so that the solvents penetrate readily. The heating should be carried through all its stages on a water bath and not on an electric hot-plate since it has been found impossible to prevent overheating on the latter.

*Separation and Determination of Cholesterol.*—After cooling, 10 cc. of chloroform are added and the flask is allowed to stand for

10 minutes; it may be shaken occasionally so that the solvent may reach any material adherent to the sides. The chloroform extract is poured through a  $5\frac{1}{2}$  cm. hardened filter into another small flask and the extraction twice repeated with 5 cc. of chloroform. If the drying and distribution of the salt have been carefully carried out, very little of the salt will break loose from the bottom of the flask during the chloroform extraction and the fatty acids are quantitatively retained. The combined chloroform extract is then evaporated down to 2 or 3 cc., poured into a 10 cc. glass-stoppered, graduated cylinder, made up to 5 cc. with chloroform washings from the flask, and the cholesterol then determined by the use of the Liebermann-Burchard reaction as follows: To the contents of the graduated cylinder made up to 5 cc. are added 1 cc. of acetic anhydride and 0.1 cc. of pure concentrated sulfuric acid, the cylinder is stoppered, and the whole well mixed. The cylinder is allowed to stand for 15 minutes at a temperature of  $20-22^{\circ}\text{C}.$ , exposed to the same light by which readings are later to be made. (The color is sensitive to light and this precaution is necessary in order to avoid changes in tint during the reading.) It is then transferred to the colorimeter cup and compared with a suitable standard similarly prepared from pure cholesterol. The standard cholesterol for this purpose should contain ordinarily 0.5 mg. of cholesterol in 5 cc. of chloroform.

*Determination of Fatty Acids.*—The residue in the small flasks after extraction with chloroform as above is treated with boiling alcohol for the extraction of the fatty acids (in the form of soaps) in the following way: 10 cc. of redistilled alcohol are added to each flask, the mixture is raised to boiling on an electric stove or water bath and kept boiling very gently for a period of 10 minutes. The hot alcohol is then poured through the small hardened filter which was used in filtering the chloroform into a 100 cc. Erlenmeyer flask. The extraction with alcohol is repeated once, using 5 cc. of alcohol, the hot extracting fluid being poured through the filter into the flask. The combined filtrates are evaporated to small volume, about 2 to 3 cc., then transferred quantitatively to a small graduated, glass-stoppered cylinder, and the flask is rinsed out with just enough alcohol to bring the volume in the cylinder up to 5 cc. 100 cc. of distilled water are next measured into a 200 cc. beaker and the alcoholic extract of the fatty acid is added with stirring

through a small funnel with the stem drawn out to form an opening about 1 mm. in diameter and extending nearly to the bottom of the beaker. The cylinder is rinsed once with the solution in the beaker and the rinsings are poured back into the beaker through the funnel. To another beaker containing 100 cc. of water are added through a pipette with stirring 5 cc. of the alcoholic standard containing 2 mg. of a mixture of oleic and palmitic acids containing 60 per cent oleic and 40 per cent palmitic acids in 95 per cent alcohol (redistilled). 10 cc. of dilute hydrochloric acid (1 part concentrated acid, 3 parts water) are added to each beaker with stirring, and after standing not less than 3 or not more than 10 minutes the solutions are compared in the nephelometer.

#### *Solutions Used.*

*Standard Fat Solution.*—A 95 per cent alcoholic solution of palmitic and oleic acids of which 5 cc. contain 2 mg. of a mixture consisting of 60 per cent oleic acid and 40 per cent palmitic acid. The standard is conveniently made by first preparing solutions of oleic and palmitic acids, each containing 200 mg. of fatty acid in 500 cc. of alcohol. For use 60 cc. of the oleic acid solution are mixed with 40 cc. of the palmitic acid solution.

*Standard Cholesterol Solution.*—This is a solution of cholesterol in chloroform containing from 0.5 to 1 mg. of cholesterol in 5 cc., depending on the cholesterol content of the blood which is being measured. For most purposes a standard containing 0.50 mg. of cholesterol in 5 cc. of solution will be found suitable. For convenience in weighing the cholesterol, a standard twenty times the strength of the final standard is prepared and this is diluted as needed.

*Sodium Hydroxide.*—Made from metallic sodium by exposing the metal, in a closed vessel containing distilled water at room temperature, over a receiver to catch the hydroxide which drips off the metal. The action is slow but the apparatus requires little attention and a strong pure hydroxide is obtained. The apparatus in use in this laboratory consists of a desiccator fitted as shown in Fig. 1.

*Chloroform.*—The chloroform used must be neutral in reaction and free from moisture and alcohol.

*Use of the Nephelometer.*—The nephelometer modified from the Duboscq colorimeter was used throughout. Since the nephelometer tubes filled with the same solution rarely give the same readings on the two sides of the instrument the standard must be adjusted before readings are made. This is done by filling both tubes with the standard solution and inserting into the instru-

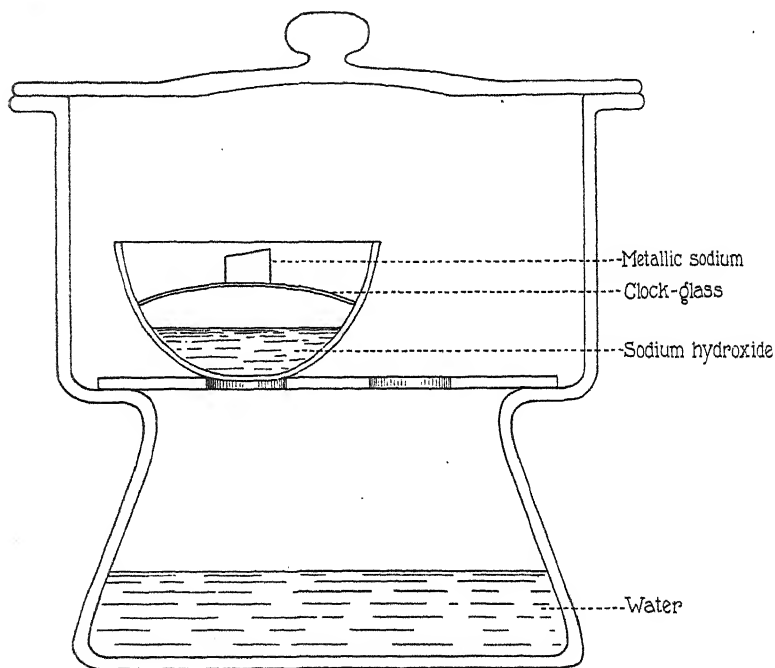


FIG. 1.

ment, after which the jacket on the right is set at 30 mm. and the jacket on the left adjusted until the two light fields are equal. This point gives the equivalent of 30 mm. on the right side and calculations are made on this basis. The tube on the right is then filled with the solution to be tested and readings are made.

Accurate determinations cannot be made if the standard and test solutions are more than 30 per cent apart. If wider differences are found the determination should be repeated using more or less of the alcohol-ether extract as required to bring the two

solutions within the limit specified. In some cases (rabbit blood) it may be necessary to use a weaker standard which is done by measuring 2 to 4 cc. of the standard fatty acid solution into one of the small graduated cylinders used for the test solution, filling to 5 cc. with alcohol, mixing, and then proceeding as with the test solution.

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## SOME HUMAN DIGESTION EXPERIMENTS WITH RAW WHITE OF EGG.

By C. G. L. WOLF.

(From Addenbrooke's Hospital, Cambridge, England.)

(Received for publication, March 7, 1922.)

There is a statement in a paper with the above title by M. S. Rose and G. MacLeod in the January number of this Journal which in the interests of the facts should be corrected.

As set forth by them:

"Wolf and Osterberg . . . . found in one case with a total intake of 23 gm. of nitrogen 70 per cent of which was derived from raw egg white, the loss of nitrogen in the feces was 41 per cent of the intake; but in another period, with a total intake of 14 gm. and 51 per cent of the nitrogen from the egg white, the loss was only 15 per cent of the total intake. . . . ."

This is not so. In the second experiment alluded to, where 444 gm. of *cooked* white of egg were consumed at breakfast, the utilization was 85 per cent, substantially the figure which these authors found in their own experiment.

This is quite a different experiment from the first, where 1,000 cc. of *uncoagulated* white of egg were taken at a single meal. Hence, there is no conflict between the results of the two experiments.

Were this last experiment repeated, I feel sure it would be found that a subject would not be able to utilize effectively so large an amount of uncooked egg white when it is administered in a single dose. This surmise receives confirmation from the results of the above named authors, when something over a third of the amount of raw egg white used in my experiments gave a difference in favor of cooked egg up to 14 per cent. In their paper they do not state whether the amounts of egg white were consumed at one meal or during the course of the day. This is a factor of some importance in estimating the effect of a large quantity of a food-stuff.



# A NOTE ON THE NUTRITIONAL ADEQUACY OF THE PROTEINS OF THE CHINESE AND GEORGIA VELVET BEANS WITH REFERENCE TO AMINO-ACID COMPOSITION.

By D. BREESE JONES, A. J. FINKS, AND H. C. WATERMAN.

(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)

(Received for publication, March 15, 1922.)

In an article entitled "The nutritive value of the proteins of the Chinese and Georgia velvet beans" Finks and Johns<sup>1</sup> showed, by feeding experiments with albino rats, that when the isolated globulins of these beans obtained by dialysis of a 10 per cent sodium chloride extract of the bean meal were fed, with or without cystine, little or no growth resulted. But, when the coagulated proteins obtained by boiling the sodium chloride extract were used, *growth at the normal rate was obtained without the addition of any supplementary amino-acid or protein whatever*. These results obtained with the coagulated proteins demonstrate that the failure to grow on either the bean meal or the unheated isolated dialyzed proteins was not due to an amino-acid deficiency. The failure to grow on the unheated dialyzed proteins was, therefore, ascribed to indigestibility. Evidence confirming this assumption was obtained by Waterman and Jones,<sup>2</sup> who by means of digestion experiments *in vitro* showed that the coagulated preparations of the proteins of the Georgia velvet bean had a decidedly higher digestibility coefficient than the unheated proteins, and that the latter became as readily digestible as the coagula when they were boiled for a short time with distilled water.

Barnett Sure in an article which appeared in a recent number of this Journal<sup>3</sup> presents the results of some experiments, which he interprets as indicating that the proteins of the Georgia velvet

<sup>1</sup> Finks, A. J., and Johns, C. O., *Am. J. Physiol.*, 1921, lvii, 61.

<sup>2</sup> Waterman, H. C., and Jones, D. B., *J. Biol. Chem.*, 1921, xlvii, 285.

<sup>3</sup> Sure, B., *J. Biol. Chem.*, 1922, I, 103.

bean are inadequate because of amino-acid deficiency and states that "cystine is unquestionably a growth-limiting factor in the proteins of the Georgia velvet bean." In this article the author refers to the publication of Waterman and Jones on the digestibility *in vitro* of the proteins of the Chinese and Georgia velvet beans, and states that we conclude that amino-acid deficiency cannot account for the failure of the velvet bean proteins to promote growth. Dr. Sure conveys the impression that our statement which he cites was based solely on the results of chemical analyses of the velvet bean proteins, and on the results obtained by the digestion experiments *in vitro*, since he makes no reference to the publication of Finks and Johns giving the results of their feeding experiments. He has also apparently overlooked our further statement immediately following the passage which he cites, that the proteins from either the Georgia or the Chinese velvet beans *gave normal growth when prepared by coagulation*.

It is the purpose of this note to correct this impression, and to make clear that our statement referring to the amino-acid adequacy of the velvet bean proteins was based primarily on the fact that growth at the normal rate was obtained when the total proteins prepared by coagulation were fed to albino rats as the sole source of protein in a diet otherwise adequate. That normal growth was obtained on the isolated velvet bean proteins after the indigestibility factor had been eliminated by heating, shows that the amino-acids essential for growth are present in these proteins in adequate amounts.

## A COLORIMETRIC METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF MAGNESIUM.\*

BY F. S. HAMMETT AND E. T. ADAMS.

(From *The Wistar Institute of Anatomy and Biology, Philadelphia.*)

(Received for publication, February 27, 1922.)

The gravimetric method for the determination of magnesium is not adequate for the estimation of the small amounts of this element present in biological material such as the ash of the bones of small animals like the rat. The microcolorimetric method devised by Kramer and Tisdall<sup>1</sup> is objectionable because of the nature and technique of the colorimetric comparisons. Inasmuch as the procedure developed by these workers is really a determination of phosphorus precipitated presumably as ammonium magnesium phosphate, it would appear as if the colorimetric method for the determination of phosphorus published by Bell and Doisy<sup>2</sup> would well be applied to the ammonium magnesium phosphate precipitate prepared according to Kramer and Tisdall<sup>1</sup> and certain advantages be gained thereby. In the first place the color reaction used by Bell and Doisy<sup>2</sup> is eminently satisfactory because the color developed is a linear function of the concentration of phosphorus in the solution. This is shown in Charts 1 and 2. The values from which Chart 1 was plotted were obtained by comparing a standard solution of monopotassium phosphate containing 0.1 mg. of phosphorus diluted to 25 cc. after the development of the color according to Bell and Doisy<sup>2</sup> and set at 20 mm. in the colorimeter, with the color developed

\* After this paper had been sent to press there appeared an abstract by Briggs (Briggs, A. P., *J. Biol. Chem.*, 1922, 1, p. xlviii) of a paper read by title only, at the New Haven meeting of the American Society of Biological Chemists, in which the colorimetric principle used in our work was briefly mentioned.

<sup>1</sup> Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlviii, 223.

<sup>2</sup> Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

by 3, 4, 5, 6, 7, and 8 cc. of a solution of ammonium magnesium phosphate containing 0.02 mg. of phosphorus per cc., all diluted to 25 cc. The values from which Chart 2 was plotted were obtained by using a monopotassium phosphate standard containing 0.5 mg. of phosphorus and 15, 20, 25, 30, 35, and 40 cc. of the solution used in the first series, all diluted to 100 cc. after the development of the blue color. In the second place the necessity for the preparation of a long series of standards is obviated when the colorimeter is used instead of the awkward comparison involved in the iron thiocyanate method.

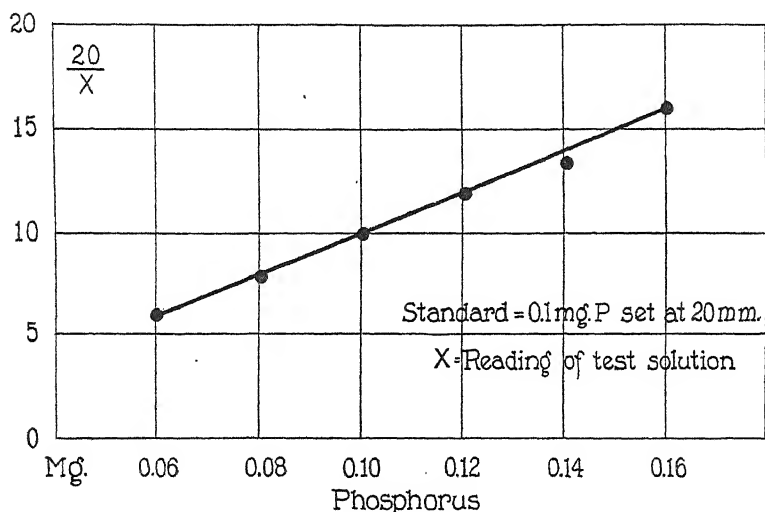


CHART 1.

In order to determine whether or not phosphorus precipitated as ammonium magnesium phosphate produces the same grade of color in the Bell and Doisy<sup>2</sup> reaction as is produced by other phosphates, 0.6017 gm. of monopotassium phosphate was put into a solution and precipitated in this form. The precipitate, after washing, was dissolved in dilute hydrochloric acid and diluted to 1 liter. 1 cc. of this solution was found to contain 0.132 mg. of phosphorus by the colorimetric method of Bell and Doisy.<sup>2</sup> The expected value was 0.136 mg. A second sample which weighed 0.5724 gm. when similarly treated gave 0.127 mg. of phosphorus per cc. with 0.129 gm. expected. By gravi-

metric analysis it was found that the monopotassium phosphate was 99.13 to 99.18 per cent pure on the basis of its phosphorus content. These results demonstrate that phosphorus precipitated as ammonium magnesium phosphate is accurately determinable colorimetrically. The slightly low results are explicable from the well known fact of the difficulty in the precipitation and recovery of all the phosphorus exactly as ammonium magnesium phosphate. This difficulty is particularly annoying in the precipitation of the small amounts of magnesium from bone ash solution according to the method of Kramer and Tisdall.

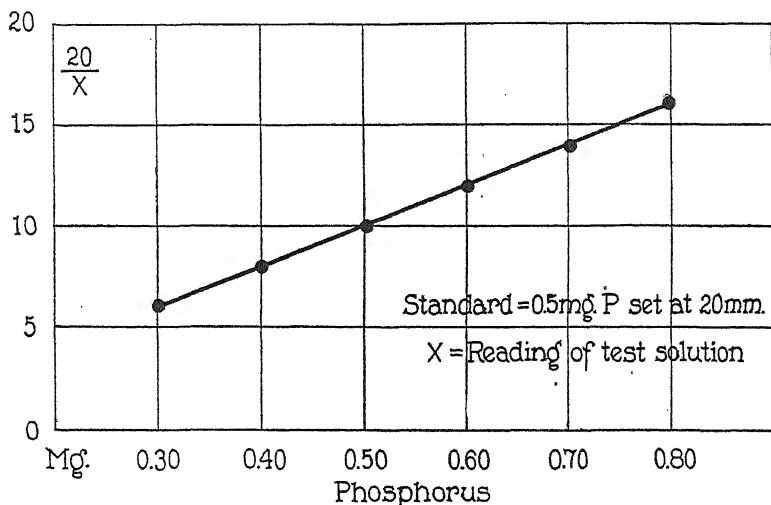


CHART 2.

Meticulous care must be exercised in this precipitation and in the subsequent filtration and washing.

In Table I are given the results of the combination of the technique of Kramer and Tisdall<sup>1</sup> and Bell and Doisy<sup>2</sup> on solutions of known phosphorus and magnesium content, with certain modifications which are given in the following detailed description of the method as we use it on solutions of bone ash.

5 or 10 cc. (depending on the weight of the bone ash obtained on incineration and the dilution of the solution made therefrom) of the clear supernatant liquid from the precipitation of calcium according to Kramer and Tisdall<sup>1</sup> are pipetted into a 30 cc.



beaker and 1 cc. of  $(\text{NH}_4)_2\text{HPO}_4$  solution, prepared according to the method of these workers, is added, drop by drop, and then 2 cc. of  $\text{NH}_4\text{OH}$ , similarly. After standing over night the precipitate is filtered through asbestos in a 27 mm. Gooch crucible with mild suction, washed ten times with 5 cc. lots of 10 per cent  $\text{NH}_4\text{OH}$  solution and twice with 90 per cent ethyl alcohol, made alkaline with  $\text{NH}_4\text{OH}$ . The crucible is replaced in the beaker and dried in the oven at  $80^\circ\text{C}$ . for a few minutes. 10 cc. of 0.01 N  $\text{HCl}$  are added to the contents of the crucible in the beaker and the whole is allowed to stand for 3 hours (it is allowable to use 10 cc. of 0.1 N  $\text{HCl}$  with 1 hour standing) at room

TABLE I.

*Magnesium Precipitated as Ammonium Magnesium Phosphate According to the Method of Kramer and Tisdall and Determined Colorimetrically by the Method for Phosphorus of Bell and Doisy.*

From $\text{KH}_2\text{PO}_4$ solution.		From $\text{Mg}_2\text{P}_2\text{O}_7$ solution in $\text{HCl}$ .	
Expected.	Obtained.*	Expected.	Obtained.*
<i>mg.</i>	<i>mg.</i>	<i>mg</i>	<i>mg</i>
0.078	0.074	0.078	0.071 0.076
0.157	0.159	0.153	0.143
0.235	0.230	0.230	0.219 0.222 0.230
0.314	0.302	0.306	0.302

\* Average of two parallel determinations.

temperature. The contents of the crucible and beaker are transferred to the test-tube and the asbestos is separated by centrifugation. 5 cc. of the supernatant liquid are pipetted into a 25 cc. graduated flask. Into a second 25 cc. graduated flask there are placed 5 cc. of a standard  $\text{KH}_2\text{PO}_4$  solution containing 0.05 mg. of phosphorus. 5 cc. of phosphate-free distilled water are added to both flasks. To each of the flasks there are added 1 cc. of the molybdic solution, 2 cc. of the hydroquinone solution, and after 5 minutes, 10 cc. of the carbonate-sulfite solution of Bell and Doisy.<sup>2</sup> The contents of the flasks are then made to the mark with distilled water, and after standing from 5 to 10

minutes, the unknown is compared with the standard in the colorimeter. The amount of phosphorus found in the test solution multiplied by  $0.7835 \times 2$  gives the amount of magnesium in the sample removed from the supernatant liquid from the calcium determination. The reduction of this value to terms of percentage in the bone varies from sample to sample according to weight of ash, dilution of the ash solution, and the amount of material used for the calcium determination. Hence no set rule can be given for the final steps in the calculation.

It is evident from the reported results that there is inherent in the method a tendency for the values to fall below the expected amounts. This may be due to incomplete precipitation or to solution during the washing. With care this loss can be held within about 3 per cent. The actual data show a mean deficiency of 3.45 per cent, with a probable error of the mean of 0.45 per cent and a standard deviation of 2.23 per cent. This is attributable to the gravimetric procedures as shown by Bell and Doisy<sup>2</sup> who also obtained lower results on gravimetric analysis. The mean deficiency of their corrected figures from the gravimetric analyses from the values obtained by the colorimetric method is 1.27 per cent, with a probable error of the mean of 0.21 per cent and a standard deviation of 0.98 per cent. When there is taken into consideration the fact that the amounts of precipitate they worked with were from 1,000 to 2,000 times the quantities we had, the deficiency and variability in our results are surprisingly low.

The combined method as outlined is obviously applicable for the estimation of small amounts of magnesium in urine, blood, and tissue extracts or incinerations. Its main advantage lies in the colorimetric comparisons which are exact and clean-cut. Its disadvantage lies in the possibility of incomplete precipitation or loss by solution during washing of the minute amounts of magnesium present in biological material. The possibility that the precipitate may not always be pure ammonium magnesium phosphate must be also considered. Nevertheless, the values obtained from the standard magnesium solution as given in the table indicate that the method of precipitation here outlined is capable of yielding reasonably satisfactory results. Attempts at precipitation in hot solution produced obviously gelatinous precipitates and this possible modification was abandoned.



# ON SOME NEW COLOR REACTIONS OF CHOLESTEROL.

By LOUIS KAHLENBERG.

(From the Department of Chemistry, University of Wisconsin, Madison.)

(Received for publication, March 7, 1922.)

In the course of some investigations on the function of cholesterol in the animal body, the results of which will be reported at a later time, I found that cholesterol was readily soluble in quite a number of anhydrous inorganic chlorides and bromides. Among these solvents are the following:  $\text{PCl}_3$ ,  $\text{AsCl}_3$ ,  $\text{AsBr}_3$ ,  $\text{SbCl}_3$ ,  $\text{SnCl}_4$ ,  $\text{SiCl}_4$ ,  $\text{SiBr}_4$ ,  $\text{TiCl}_4$ ,  $\text{SOCl}_2$ ,  $\text{POCl}_3$ ,  $\text{SeOCl}_2$ , and  $\text{CBr}_4$ . Since colorless solutions are obtained when cholesterol is dissolved in the ordinary solvents (such as chloroform, carbon tetrachloride, carbon disulfide, alcohol, ether, and acetone), one would naturally expect the corresponding solutions of cholesterol in the above named anhydrous chlorides to be colorless. Such is indeed the case in many of these solvents. However, important exceptions to this rule occur, and to describe these is the special purpose of this article.<sup>1</sup>

## *Materials Used.*

Several samples of cholesterol were used in making the investigation:

1. A fine colorless sample of well formed odorless crystals of Kahlbaum's make. It melted at  $148.45^\circ\text{C}$ ., corrected, and after recrystallizing from absolute alcohol, it still had exactly the same melting point.

2. A sample of Merck's manufacture. The crystals were small but fairly well developed. They showed a slight tendency to stick together, had a faint yellowish tinge, and a slight odor suggesting that of lanolin. This sample melted at  $143.0^\circ\text{C}$ ., corrected. On recrystallization from absolute alcohol, the melting point rose to  $147.95^\circ\text{C}$ .

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<sup>1</sup> The investigation of the properties of the colorless solutions of cholesterol in anhydrous chlorides and many other solvents is in progress in this laboratory and it is hoped to have the experimental data ready for publication soon.

3. Human gall-stones were pulverized and extracted with ether. After evaporation of the ether from the clear extract, the residue was recrystallized several times from absolute alcohol and finally from acetone. The crystals melted at  $148.5^{\circ}\text{C}$ . They were colorless and odorless and did not cling together.

4. Cholesterol was prepared<sup>2</sup> from the brains of normal young pigs raised on the University Farm. These brains were well washed and freed from all bloody matter. They were then reduced to a pulp and mixed with plaster of Paris. After drying over night, the mass was broken into pieces of about the size of a grain of wheat and transferred to percolators, the cholesterol being extracted by means of acetone at room temperature. The crystals obtained from this acetone solution were recrystallized several times from absolute alcohol. They were almost white, but they always showed a slight tendency to cling together. Moreover, they always had a faint odor suggesting hog's lard. When first obtained from the acetone solution, the sample melted at  $146.0^{\circ}\text{C}$ . After recrystallizing twice from absolute alcohol, the melting point rose to  $148.15^{\circ}$  and on further recrystallization, crystals melting at  $148.5^{\circ}$  were obtained.

5. Cholesterol was similarly prepared from pigs' brains obtained from Swift and Company, Chicago, through the kindness of Dr. W. D. Richardson, to whom I wish to express my thanks. The final crystals melted at  $148.4^{\circ}\text{C}$ ., corrected.

6. Cholesterol was finally also prepared from the spinal cords of pigs by the same method. When first obtained from the acetone solution the crystals melted at  $144.5^{\circ}$ . On twice recrystallizing from absolute alcohol, the melting point rose to  $144.15^{\circ}$ , and a final recrystallization yielded a product melting at  $148.5^{\circ}\text{C}$ ., corrected.

In some of the experiments described below, lanolin was tested as to its behavior toward the solvents and several samples of lanolin were employed. Two of these were obtained from Lehn and Fink, one was from Eimer and Amend, another from Merck, and a fifth one was an old sample that had been in the laboratory in a well stoppered bottle for over 10 years. All of these samples were of U.S.P. quality, and they all showed the same behavior toward each of the solvents employed.

The solvents used were all of the highest purity manufactured by either Kahlbaum, Merck, Schuchardt, or Baker and Adamson, except the silicon tetrabromide which was prepared under my direction by one of my pupils, Mr. E. H. Zobel. It boiled very constantly at  $148.4^{\circ}\text{C}$ . at 725 mm. The selenium oxychloride was a sample kindly furnished by Dr. Victor Lenher.

<sup>2</sup> Compare the method of Rosenheim, O., *J. Physiol.*, 1906, xxxiv, 104.

## EXPERIMENTAL OBSERVATIONS.

The behavior of cholesterol when dissolved in each of the solvents employed was found to be as follows:

In  $\text{PCl}_3$ ,  $\text{POCl}_3$ ,  $\text{SnCl}_4$ ,  $\text{SiCl}_4$ ,  $\text{SiBr}_4$ , and  $\text{CBr}_4$ , all of the six samples of cholesterol above mentioned dissolved and yielded colorless solutions.

In  $\text{SOCl}_2$ , cholesterol prepared from human gall-stones (Sample 3) and that prepared from brain or nerve tissues (Samples 1, 4, 5, and 6), dissolved readily at room temperatures. The solution is of a beautiful wine color, changing to a brown on standing. On heating the wine-colored solution, it rapidly changes to brown, which at times has a very faint greenish hue.<sup>3</sup> When Merck's cholesterol (Sample 2) was dissolved in  $\text{SOCl}_2$  a beautiful wine-colored solution was also obtained. This changed to brown on standing. On gentle heating, the wine-colored solution changed to a brilliant dark green, which then passed over into brown. The color of these solutions is not discharged on adding benzene. When lanolin was dissolved in  $\text{SOCl}_2$  a reddish brown solution was obtained which changed to brown on standing. On heating, the reddish brown solution passed over into brown more rapidly. On long standing, all of the solution in  $\text{SOCl}_2$  became very dark, almost black, in appearance.

When crystals of cholesterol (Samples 1 to 6 inclusive) are rubbed in a mortar with crystals of  $\text{SbCl}_3$ , the mixture which is colorless at first, turns pink, then flesh color, gradually becoming more and more brownish in hue. On long standing, the crystals completely liquefy each other forming a reddish brown syrupy mass. In molten  $\text{SbCl}_3$ , cholesterol readily dissolves, yielding at once a rich dark brown solution as might have been expected from what has just been said. When lanolin is rubbed with  $\text{SbCl}_3$  crystals in a mortar, a dark brown paste is obtained, and when lanolin is introduced into molten  $\text{SbCl}_3$  a rich dark brown solution is formed.

<sup>3</sup> In 1904, Diels and Abderhalden (Diels, O., and Abderhalden, E., *Ber. chem. Ges.*, 1904, xxxvii, 3102) prepared cholesterylchloride by the action of cholesterol on  $\text{SOCl}_2$ . While they must have observed the color changes that take place, they did not record them.

$\text{SbCl}_5$  dissolves neither cholesterol nor lanolin. With cholesterol there is formed a chocolate brown, gummy substance, while with lanolin  $\text{SbCl}_5$  forms a very dark brown smeary mass.

In  $\text{TiCl}_4$ , cholesterol dissolves readily at room temperatures. All the cholesterol samples behaved alike toward this reagent, yielding a yellow solution which turned brown on adding more cholesterol. On heating this solution it became markedly more red in hue, developing a wine color on boiling and finally changing to a very dark brown. The solution of lanolin in  $\text{TiCl}_4$  is a rich dark brown at room temperatures, becoming redder and darker on heating, turning to a wine color, and finally to a rich dark brown. The color of the solutions in  $\text{TiCl}_4$  is not discharged by adding benzene.

In  $\text{SeOCl}_2$ , all of the samples of cholesterol yielded a brilliant, dark slightly yellowish red solution which changed to a pale yellow on dilution with benzene. Phytosterol yielded a yellowish red solution in  $\text{SeOCl}_2$ , while lanolin gave a reddish brown solution in that solvent. The reactions in  $\text{SeOCl}_2$  are not specially characteristic.

*By far the most interesting behavior is that observed when cholesterol is dissolved in  $\text{AsCl}_3$ . Indeed, it is the discovery of the behavior in that solvent which really led to this entire investigation, for it was found that the color of solutions of cholesterol in  $\text{AsCl}_3$  may be used to distinguish between cholesterol and isocholesterol, and also between these on the one hand and phytosterol on the other.*

When cholesterol from nerve tissues or gall-stones (Samples 1, 3, 4, 5, and 6) is dissolved in  $\text{AsCl}_3$  at room temperatures, the solution turns pink almost immediately, becoming redder in the course of a few minutes, and finally on standing, gradually assuming a pretty cherry-red color. If the solution is gently heated, the appearance of the cherry-red color is greatly hastened. However, if  $\text{AsCl}_3$  is first chilled to  $0^\circ\text{C}$ . or lower, cholesterol may be dissolved in it, yielding a solution that is practically colorless, and it may be kept so at low temperatures for sometime, for under these conditions, the pink color develops very slowly. On the other hand, if a solution of cholesterol in  $\text{AsCl}_3$  be heated, it changes very rapidly to the cherry-red hue and on continued boiling this goes over into a dirty green, which, however, on cooling and standing passes back into the cherry-red again. How-

ever, when once even a pink color is developed, the solution of cholesterol in  $\text{AsCl}_3$  will not become colorless again though it be kept chilled with a mixture of ice and salt; that is to say, the color reaction is not reversible. Solutions of 1 per cent or much less of cholesterol in  $\text{AsCl}_3$  enable one to make all of these observations. In fact, it is better to work with such solutions rather than more concentrated ones, for the latter give correspondingly deeper colorations that are not so well followed in their change of hue. The pink solution of cholesterol in  $\text{AsCl}_3$  changing to a deep cherry-red color is very characteristic of cholesterol, and it may be used to distinguish cholesterol from phytosterol, for the latter dissolves in  $\text{AsCl}_3$ , yielding colorless solutions which remain colorless on standing and even on boiling. Six different beautifully crystalline samples of phytosterol<sup>4</sup> were thus tested and all of them dissolved readily in  $\text{AsCl}_3$ , yielding colorless solutions.

When Sample 2 of cholesterol was dissolved in  $\text{AsCl}_3$  a markedly different behavior from that of any of the other samples was observed. The dilute solution was blue, becoming slightly violet on standing; on adding more cholesterol, smalt-blue; on warming this solution, greenish blue; on boiling, green; then very dark green. On standing, this hot solution became muddy and darker, losing its green hue. However, on chilling the test-tube under the tap, the color returned. It was purplish, however, gradually becoming dark reddish brown. On standing over night in the cold below  $0^\circ\text{C}$ . out of doors it took on a dark permanganate color. As already stated the behavior of the  $\text{AsCl}_3$  solution of this sample of cholesterol (No. 2) was quite different from that observed with any of the other samples of cholesterol, for in no other case was a cobalt blue solution obtained. The change of this blue to violet and green was also characteristic of Sample 2. Since it had an odor of lanolin (from which it had evidently been prepared), a sample of lanolin was dissolved in  $\text{AsCl}_3$ . The resulting solution was green, not blue, it remained dark green even on boiling. This lanolin was anhydrous U.S.P. obtained from Lehn and Fink. A hydrous sample of lanolin (*i.e.* the white layer under the brownish

<sup>4</sup> These samples of phytosterol were kindly put at my disposal by Dr. H. A. Langenhan of the Department of Pharmacy of the University of Wisconsin. He had personally prepared them. I desire here to express my indebtedness to him.



layer which forms on top) when dissolved in  $\text{AsCl}_3$  yielded a violet solution. On heating this solution, it became bluish green, and on boiling, dark olive-green. Evidently the aqueous lanolin, then, is the cause of the violet color. Again, a sample of lanolin of the hydrous variety (taken from the top brown layer this time) when dissolved in  $\text{AsCl}_3$  yielded a blue solution. The color was a brilliant cobalt blue which turned to violet in the course of about 2 minutes. On heating, the solution turned bluish green, and on boiling, dark green. If a sample of anhydrous lanolin is first heated with water so as to make it hydrous, this hydrous sample dissolves in  $\text{AsCl}_3$ , yielding the violet color, which changes to bluish green and finally to dirty green on heating. If anhydrous lanolin is first saponified by heating with concentrated alcoholic potassium or sodium hydroxide and then boiled up with water, the insoluble portion obtained, yields a deep cobalt blue solution when dissolved in  $\text{AsCl}_3$ .

From these experiments it is evident that anhydrous lanolin yields a green solution with  $\text{AsCl}_3$ , hydrous lanolin a violet solution, and "rancid" hydrous lanolin (*i.e.* the top brownish layer on an old sample of hydrous lanolin) yields a cobalt blue. This cobalt blue solution is also obtained when lanolin is saponified and the released cholesterol is treated with  $\text{AsCl}_3$ . Now since lanolin is known to contain isocholesterol, and since from what has been said above, brain cholesterol or gall-stone cholesterol yields a pink and then a cherry-red solution with  $\text{AsCl}_3$ , it was concluded that the cobalt blue solution with  $\text{AsCl}_3$ , results when isocholesterol is dissolved in  $\text{AsCl}_3$ , for there would be some free isocholesterol in rancid lanolin and also in the insoluble matter remaining after saponifying lanolin. To test this further, isocholesterol was actually prepared by saponifying lanolin with alcoholic potassium extracting the mixture of cholesterol and isocholesterol from the soap by means of ether, and then forming the benzoates and separating the crystals of cholesterol benzoate from those of isocholesterol benzoate. From the latter compound isocholesterol was then released by saponification. It was found that when this isocholesterol was dissolved in  $\text{AsCl}_3$  a cobalt blue solution was formed, showing that the blue color indicates the presence of isocholesterol.

On the other hand, cholesterol itself (from brain or gall-stone material) yields a pink solution in  $\text{AsCl}_3$  which changes to cherry-red. The mixture of brain cholesterol and isocholesterol yields a blue solution which changes to violet on standing, showing that the blue compound masks the red reaction. On boiling, however, a redder solution is obtained in any case. With a solution containing about a gram of cholesterol from lanolin (no attempt having been made to separate this cholesterol from the isocholesterol) in 5 cc. of  $\text{AsCl}_3$ , a solution having the color of an aqueous potassium permanganate solution was obtained.

The attempt was made to isolate the colored compound from the  $\text{AsCl}_3$  solution of cholesterol. To this end, 1 gm. of cholesterol was dissolved in 5 cc. of  $\text{AsCl}_3$ . Complete solution readily took place at room temperature. The color of this solution was at first violet, and as it became more concentrated it turned purple. Then another gram of cholesterol crystals was added. It, too, almost dissolved at room temperature, but the solution was not clear. It was heated to boiling, when it became clear. On standing, it assumed a dark cherry-red color within about 5 minutes. On chilling, the solution became somewhat cloudy, *i.e.* it remained red but it was not clear, having more the appearance of blood. On chilling this solution with ice, nothing separated out, though the solution became markedly more viscous. Even on chilling with an ice and salt mixture, nothing separated out. However, on standing over night out of doors while the temperature was about  $-10^\circ\text{C}.$ , crystals were found the next morning. These crystals formed a top layer completely covering the liquid. This layer was punctured and the liquid was drained off in the cold. The crystals were then placed on a cold tile and as the reddish mother liquor drained from them, the crystals appeared white. They proved to be cholesterol. They were weighed, and it was found that thus nearly 100 per cent of the cholesterol used could be recovered from the solution, which remained about permanganate color in the cold, even though it contained but little cholesterol under these conditions. When cholesterol is dissolved in  $\text{AsCl}_3$ , therefore, we get a colored solution, but from this cholesterol may again be separated out practically quantitatively on standing at low temperatures.

When to a colored solution of cholesterol or ischolesterol in  $\text{AsCl}_3$ , a solvent like benzene, toluene, or chloroform, is added, the color is discharged; *i.e.*, the solution becomes colorless. If water is added to a colored solution of cholesterol or ischolesterol in  $\text{AsCl}_3$ ,  $\text{As}_2\text{O}_3$  and cholesterol are thrown out, the entire mixture becoming colorless as one would expect. If instead of water, sufficiently strong hydrochloric acid is used, the arsenic remains in solution, but the color disappears, being retained for a time, however, by globules of cholesterol which separate out and remain colored for a time. On long standing, however, the color disappears even from these, the arsenic passing into the aqueous hydrochloric acid solution.

By dissolving  $\text{As}_2\text{O}_3$  in a concentrated aqueous solution of  $\text{HCl}$ , and adding cholesterol to this hot, concentrated solution and boiling for a few moments and then allowing to stand, a colored liquid layer separates out on the bottom of the aqueous solution. This layer is red when brain cholesterol is used and violet-colored when ischolesterol is employed. On long standing in the cold, the colored layer loses its color due to gradual hydrolysis, cholesterol separating out.

The use of these color reactions with  $\text{AsCl}_3$  in testing for cholesterol and ischolesterol in fats, oils, etc., is already in progress in this laboratory.

In the course of this investigation it was found that  $\text{AsCl}_3$  also gives characteristic color reactions with certain other organic compounds, notably those of the terpene series or derivatives thereof, a fact which supports the view that cholesterol itself is a terpene derivative.<sup>5</sup> Further investigation in this direction is in progress here.

#### SUMMARY.

1. Cholesterol, ischolesterol, phytosterol, and lanolin dissolve in quite a number of anhydrous inorganic chlorides. In  $\text{PCl}_3$ ,  $\text{POCl}_3$ ,  $\text{SnCl}_4$ ,  $\text{SiCl}_4$ ,  $\text{SiBr}_4$ , and  $\text{CBr}_4$ , these solutions are all colorless.

2. In  $\text{SOCl}_2$ ,  $\text{TiCl}_4$ ,  $\text{SeOCl}_2$ ,  $\text{SbCl}_3$ , and  $\text{AsBr}_3$ , colored solutions are formed. With  $\text{SbCl}_5$ , brownish masses are obtained. None of these reactions are, however, sufficiently characteristic to enable

<sup>5</sup> Compare Windaus, A., *Ber. chem. Ges.*, 1915, xlvii, 1065; 1917, 1, 133.

one to use them to distinguish between the different substances dissolved.

3. In  $\text{AsCl}_3$ , brain cholesterol or gall-stone cholesterol dissolves, yielding a pink solution which gradually turns to a bright cherry-red on standing, more rapidly on heating. Isocholesterol yields a cobalt blue solution which changes to violet, then to purple, dark red, and dark green on standing; more rapidly on heating. Phytosterol yields colorless solutions in  $\text{AsCl}_3$ . These reactions enable one to distinguish between cholesterol, isocholesterol, and phytosterol. The color of all these solutions is discharged by adding solvents like benzene, toluene, or chloroform.

4. Concentrated aqueous solutions of  $\text{AsCl}_3$  (in the presence of a large excess of  $\text{HCl}$ ) also give the color reactions with cholesterol or isocholesterol on boiling. On standing, the colored layers, which separate out on the bottom of the test-tubes gradually suffer hydrolysis, becoming colorless, the sterol separating out. The reaction is, however, best obtained by dissolving cholesterol or isocholesterol in anhydrous  $\text{AsCl}_3$ .

5. From the concentrated colored solutions of cholesterol in  $\text{AsCl}_3$ , cholesterol separates out practically quantitatively on standing at sufficiently low temperatures.



# THE SYNTHESIS OF $\alpha$ -HYDROXYISOPENTACOSANIC ACID AND ITS BEARING ON THE STRUCTURE OF CEREBRONIC ACID.

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Thudichum<sup>1</sup> was undoubtedly the first to isolate cerebronic acid. Unfortunately, he failed to recognize it as an oxy-acid and named it neurostearic acid. Thierfelder,<sup>2</sup> in 1904, recognized that cerebronic acid was an oxy-acid having the elementary composition  $C_{25}H_{50}O_3$ , but he gave no further details of its structure. In 1912, Levene and Jacobs<sup>3</sup> established the allocation of the hydroxyl group in the  $\alpha$ -position to the carboxyl. The same conclusion was reached by Brigl in 1915. Regarding the further details of the structure of the acid two rival views were expressed. Levene and Jacobs<sup>3</sup> and Levene and West<sup>4</sup> had obtained on oxidation of cerebronic acid an acid of elementary composition,  $C_{24}H_{48}O_2$ . This acid melted at  $81^\circ C$ . The hydrocarbon obtained from this acid melted at  $51-52^\circ C$ . Since lignoceric acid is known to melt at  $81^\circ C$ ., and the melting point of the hydrocarbon obtained from it was found to be  $51-51.5^\circ C$ ., it was thought probable that the acid obtained on oxidation of cerebronic acid was lignoceric acid. Hence the structure of cerebronic acid was correlated to that of lignoceric acid.

Brigl<sup>5</sup> working under the direction of Thierfelder came to the conclusion that cerebronic acid had the structure of a normal acid. This theory was based on the following evidence. A normal  $\alpha$ -hydroxypentacosanic acid was prepared and was found to melt

<sup>1</sup> Thudichum, J. L. W., *Die Chemische Konstitution des Gehirns des Menschen und der Tiere*, Tübingen, 1901, 194, 195.

<sup>2</sup> Thierfelder, H., *Z. physiol. Chem.*, 1904-05, xliii, 21.

<sup>3</sup> Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 381.

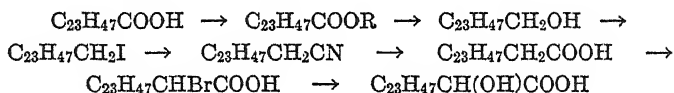
<sup>4</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913, xiv, 257.

<sup>5</sup> Brigl, P., *Z. physiol. Chem.*, 1915, xcv, 161.

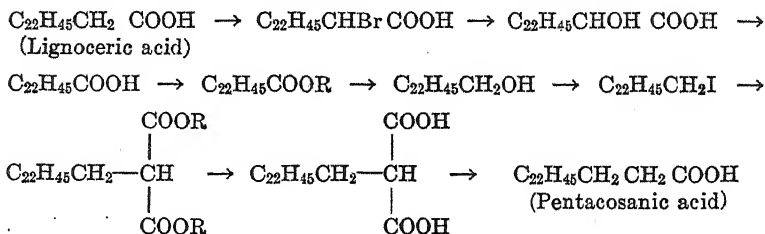
at 102–104°C. An active cerebronic acid melting at 97–100°C. was inactivated. (Brigl does not give any experimental data regarding the method of racemization.) The melting point of the inactive acid remained unchanged. The mixed melting point of the synthetic acid and the inactivated natural acid was 98–100°C.

For conclusive proof of the view of Levene and his coworkers, there still lacked the proof through synthesis.  $\alpha$ -Hydroxypentacosanic acid was prepared from lignoceric acid in order to compare it with the *dl*-cerebronic acid. The question naturally arose as to the method of racemization of cerebronic acid. The optical rotation observed on cerebronic acid is very low; namely,  $[\alpha]_D^{20} = +4.16^\circ$ . Admixture of small proportions of this acid with the *dl*-form could scarcely be discovered. Hence it was thought of converting cerebronic acid into  $\alpha$ -halogen acid which could then be reduced to a pentacosanic acid and this again converted into a *dl*-hydroxypentacosanic acid. It was, however, found impracticable to follow this plan and, therefore, cerebronic acid was oxidized to the acid  $C_{24}H_{48}O_2$ . This was then converted into hydroxypentacosanic acid.

The intermediate steps in the conversion of lignoceric acid and of the tetracosanic acid, from cerebronic acid, into  $\alpha$ -hydroxypentacosanic acid, were identical, and as follows:



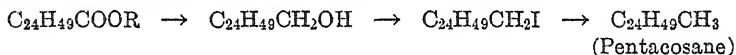
The melting points of the corresponding substances of each series, as well as the melting points of their mixtures, were identical. On the basis of these data, the evidence is conclusive that cerebronic acid is related to lignoceric acid. In order to render the proof more rigorous and to give greater confidence in the value of the melting points it was concluded to convert lignoceric acid into cerebronic acid by a second series of reactions as follows:



The melting point of this acid was identical with the corresponding acid obtained by the first process.

Another method commonly used for the identification of a fatty acid is its conversion into the corresponding hydrocarbon. Levene and Jacobs<sup>3</sup> and Levene and West<sup>4</sup> have converted cerebronic acid into a pentacosane by heating the acid with hydriodic acid. The product thus obtained melted at 53–54°C. However, it was realized that this method of preparing the hydrocarbon was not satisfactory. Therefore, lignoceric and the tetracosanic acids obtained from cerebronic acid, respectively, were converted into pentacosanes. Each melted at 51–51.5°C. It still seemed desirable to compare the two pentacosanes.

The pentacosanic acids prepared in the course of this work offered a convenient material for preparation of the pentacosanes corresponding to the pentacosanic acids from cerebronic and lignoceric acids by the following set of reactions:



Every intermediate step in this set of reactions involves very mild treatment and every intermediate product can be readily purified. The melting points of the two hydrocarbons were identical; namely, 56–56.5°C. Again, on the ground of the melting points of the pentacosanes the relationship of cerebronic to lignoceric acid is confirmed. In connection with the pentacosane here described attention is called to the normal pentacosane prepared by Brigl.<sup>5</sup> The substance was prepared by the old Kraft method from the cetyloctylketone and melted at 55.5–56°C. If this melting point is correct for the normal pentacosane then the conversion of fatty acids with a very high number of carbon atoms to their respective hydrocarbons will have little value for the identification of fatty acids.

However, it is doubtful whether it is justifiable to rely on the absolute purity of the hydrocarbons prepared by Kraft's method from ketones. We intend to prepare normal pentacosane from normal pentacosanic acid by the same set of reactions as were used for the preparation of the isopentacosanes herein described.

The following table records all the melting points of the intermediate and final products prepared, respectively, from lignoceric acid and from tetracosanic acid obtained from cerebronic acid. (M.P. 99°C.,  $[\alpha]_D^{20} = +3.5^\circ$  and M.P. 99.5°C.,  $[\alpha]_D^{20} = +3.8^\circ$ .)



Derivative of fatty acid.	M.P. of derivative of lignoceric acid from peanut oil.	M.P. of derivative from <i>dl</i> -cerebronic acid.	M.P. of derivative of lignoceric acid from peanut oil.	M.P. of mixture.
	°C.	°C.	°C.	°C.
$\alpha$ -Bromolignoceric acid.....	68-69			
$\alpha$ -Hydroxylignoceric acid.....	91-92			
Isotricosanic acid.....	73.5			
Ethyl isotricosanate.....	55.5			
Isotricosyl alcohol.....	69			
Isotricosyl iodide.....	48			
Diethyl isotricosyl-malonate.....	52.5			
Isotricosyl-malonic acid.....	111			
Lignoceric acid.....		80.5	81	80.5
Ethyl lignocerate.....		55	55	55
Lignoceryl alcohol.....		72	72	72
Lignoceryl iodide.....		48	48	48
Lignoceryl cyanide.....		56.5	56.5	56.5
Isopentacosanic acid.....	78.5	78.5	78.5	78.5
$\alpha$ -Bromoisopentacosanic acid. ....		70	70	70
$\alpha$ -Hydroxyisopentacosanic acid....		92.5	92.5	92.5
Ethyl isopentacosanate.....		57	57	57
Isopentacosyl alcohol.....		75	75	75
Isopentacosyl iodide.....		51.5	51.5	51.5
Isopentacosane.....		56	56.5	56.5

From this table it is seen that the melting points of the *dl*-cerebronic acid prepared either from lignoceric acid or from tetracosanic acid obtained from cerebronic acid, melted at 92.5°C. The original cerebronic acid had approximately the same melting point as the cerebronic acids described by Thierfelder<sup>2</sup> and by Brigl.<sup>5</sup> The melting point of the inactivated cerebronic acid is given by Brigl 97-100°C. From this he attributes to cerebronic acid the structure of the normal acid for which the melting point is 102-104°C. It is evident on the basis of the results described here that Brigl has not succeeded in inactivating cerebronic acid by his process. In fact, the observation that the melting point lagged between 97-100°C. casts a suspicion on the purity of his substance. Also the normal hydroxypentacosanic acid, if it were pure, should be expected to melt more sharply than at 102-104°C. In a word, the data furnished by Brigl may be entirely disregarded in considering the structure of cerebronic acid. As evident from the present work this is  *$\alpha$ -hydroxylignoceropentacosanic acid*.

There is one point that needs further investigation as a result of the present work. Thudichum, who was the first to isolate from phrenosin an acid having the elementary composition of hydroxypentacosanic acid, found its melting point at 84–85°C. Levene and Jacobs and Levene and West have also observed the same melting point of hydroxypentacosanic acid which was optically inactive. The low melting acid was regarded as the inactive cerebronic acid, perhaps in an impure state. Brigl<sup>15</sup> later expressed the view that the low melting acid was an isomer of cerebronic acid. Since the melting point of the *dl*-form of cerebronic acid is now established at 92.5°C., the question of the nature of the low melting acid needs further investigation.

#### EXPERIMENTAL.

In the following experiments, the melting points recorded were determined with a standardized Anschütz thermometer. The bath was continuously stirred and was heated by a burner regulated to raise the temperature 1° in 6 seconds.

Before analysis, each substance was dried to constant weight under a pressure of 0.5 mm. at the temperature of boiling chloroform.

The acid numbers were determined in mixtures of methyl alcohol (50 cc.) and benzene (25 cc.), using phenolphthalein as indicator. They are recorded as molecular weights.

*Lignoceric Acid*.—The acid from peanut oil which was used as the starting point for the following series of experiments was analytically pure and melted at 81°C. as given in the literature.

0.7286 gm. substance required 19.70 cc. 0.1 N NaOH.

0.1004 " " : 0.2880 gm. CO<sub>2</sub> and 0.1166 gm. H<sub>2</sub>O.

C<sub>24</sub>H<sub>48</sub>O<sub>2</sub>. Calculated. C 78.26, H 13.04, Mol. wt. 368.

Found. " 78.23, " 13.00, " " 370.

*α-Bromolignoceric Acid*.—Dry, analytically pure lignoceric acid (50 gm.) was mixed with red phosphorus (6 gm.), melted on the water bath, and bromine (135 gm.) cautiously added. The heating was then continued for 6 to 7 hours. The hot, liquid acid bromide was decomposed in cold water and the *α*-bromolignoceric acid filtered off. In order to remove most of the water, the acid was dissolved in ether and the solution filtered. The ether was

removed by distillation and the residue twice crystallized from 50–60° petroleum ether. The acid was then analytically pure and melted at 68–69°C. Meyer, Brod, and Soyka,<sup>6</sup> after extensive purification, found a melting point of 68.5°C. The yield was 80 per cent of the theoretical.

0.1000 gm. substance: 0.2360 gm. CO<sub>2</sub> and 0.0958 gm. H<sub>2</sub>O.

C<sub>24</sub>H<sub>47</sub>O<sub>2</sub>Br. Calculated. C 64.39, H 10.59.

Found. " 64.36, " 10.72.

*α-Hydroxylignoceric Acid.*—*α*-Bromolignoceric acid (50 gm.) was hydrolyzed by heating on the water bath for 30 hours with 12 per cent sodium hydroxide solution (1,200 cc.). The mixture was vigorously stirred. When the operation was finished, the soap was filtered off and heated with dilute hydrochloric acid on the water bath until it liquefied. One crystallization from a liter of acetone usually sufficed to give an analytically pure product. The melting point was 91–92°C. as given by Meyer, Brod, and Soyka.<sup>6</sup> The yield was 95 per cent of the theoretical.

0.1035 gm. substance: 0.2854 gm. CO<sub>2</sub> and 0.1176 gm. H<sub>2</sub>O.

C<sub>24</sub>H<sub>48</sub>O<sub>2</sub>. Calculated. C 74.92, H 12.58.

Found. " 74.99, " 12.68.

*Isotricosanic Acid.*—This acid was prepared by the oxidation of *α*-hydroxylignoceric acid (50 gm.) in boiling acetone solution, with potassium permanganate, essentially as described by Levene and West<sup>7</sup> for the conversion of cerebronic into lignoceric acid. The finely divided permanganate (about 50 gm.) was dissolved in 2 liters of warm acetone. After all the permanganate had been introduced, the mixture was boiled until the pink color had disappeared. The solution was then cooled and filtered and the potassium salt of the isotricosanic acid separated from the manganese dioxide by three extractions with boiling 99.5 per cent alcohol (1 liter each). The salt which separated on cooling was decomposed by heating with dilute hydrochloric acid. After crystallizing the product from acetone and then from 99.5 per cent alcohol, it was analytically pure. The yield was 80 per cent of the theoretical. The acid melted at 73.5°C. after passing over the lead salt.

<sup>6</sup> Meyer, H., Brod, L., and Soyka, W., *Monatsch. Chem.*, 1913, xxxiv, 1133.

<sup>7</sup> Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913–14, xvi, 475.

0.8048 gm. substance required 22.60 cc. 0.1 N NaOH.

0.0999 " " : 0.2854 gm. CO<sub>2</sub> and 0.1162 gm. H<sub>2</sub>O.

C<sub>23</sub>H<sub>46</sub>O<sub>2</sub>. Calculated. C 77.97, H 12.99, Mol. wt. 354.

Found. " 77.91, " 13.02, " " 356.

*Ethyl Isotricosanate*.—Isotricosanic acid (30 gm.) was dissolved in absolute alcohol (200 cc.) together with sulfuric acid (9.5 gm.) and the solution boiled for 10 hours under a reflux condenser. The ester separated on cooling. One crystallization from 99.5 per cent alcohol gave an analytically pure product. The melting point of the ester was 55.5°C. The yield was 92 per cent of the theoretical.

0.1010 gm. substance: 0.2909 gm. CO<sub>2</sub> and 0.1176 gm. H<sub>2</sub>O.

C<sub>25</sub>H<sub>50</sub>O<sub>2</sub>. Calculated. C 78.53, H 13.09.

Found. " 78.52, " 13.05.

*Isotricosyl Alcohol*.—The alcohol was prepared by the reduction of the corresponding ester in the manner described by Levene and Cretcher.<sup>8</sup> Because of the comparatively slight solubility of the ester, it was convenient to work with 10 gm. lots dissolved in a mixture of absolute alcohol (30 cc.) and dry toluene (15 cc.). Six times the calculated quantity of sodium was used. When the reduction was finished, the ethyl alcohol and toluene were removed in a current of steam, the soap and isotricosyl alcohol filtered off and extracted repeatedly with acetone. On cooling the acetone solution, the alcohol separated. It was purified by two further crystallizations from acetone and then melted at 69°C. The yield of analytically pure material was 76 per cent of the theoretical. Practically all of the remainder was recovered as isotricosanic acid.

0.1000 gm. substance: 0.2968 gm. CO<sub>2</sub> and 0.1286 gm. H<sub>2</sub>O.

C<sub>23</sub>H<sub>48</sub>O. Calculated. C 81.09, H 14.21.

Found. " 80.94, " 14.39.

*Isotricosyl Iodide*.—The alcohol (20 gm.) was heated for 1 hour at 180°C. with 1.1 equivalents of iodine and an excess of red phosphorus. The product was twice crystallized from acetone and was then analytically pure. The iodide melted at 48°C. The yield was 95 per cent of the theoretical.

<sup>8</sup> Levene, P. A., and Cretcher, L. H., Jr., *J. Biol. Chem.*, 1918, xxxiii, 505.

0.2024 gm. substance: 0.1046 gm. AgI (Carius).

$C_{25}H_{47}I$ . Calculated. I 28.22.

Found. " 27.92.

*Diethyl Isotricosyl-Malonate*.—Isotricosyl iodide (20 gm.) was dissolved in absolute alcohol containing one equivalent of sodium ethylate and two equivalents of diethyl malonate. The solution was boiled on the water bath until neutral. The ester which separated on cooling was contaminated by a small quantity of isotricosyl iodide which was not entirely removed after a number of crystallizations. It melted at  $52.5^{\circ}\text{C}$ .

0.1000 gm. substance: 0.2724 gm.  $\text{CO}_2$  and 0.1084 gm.  $\text{H}_2\text{O}$ .

$C_{30}H_{58}O_4$ . Calculated. C 74.69, H 12.03.

Found. " 74.28, " 12.13.

*Isotricosyl-Malonic Acid*.—Crude isotricosyl-malonic ester was saponified by heating over night with a large excess of alcoholic sodium hydroxide on the water bath. The alcohol was then removed, the residue diluted with water, the crude soap filtered off and extracted with acetone. The soap was decomposed by heating with dilute hydrochloric acid on the water bath and the acid passed over the lead salt. It melted at  $111^{\circ}\text{C}$ . Nearly pure isotricosyl alcohol was recovered as a by-product from the acetone extract of the sodium salt.

0.0996 gm. substance: 0.2680 gm.  $\text{CO}_2$  and 0.1026 gm.  $\text{H}_2\text{O}$ .

$C_{26}H_{50}O_4$ . Calculated. C 73.24, H 11.74.

Found. " 73.38, " 11.53.

*Isopentacosanic Acid*.—The substituted malonic acid was heated at  $180^{\circ}\text{C}$ . as long as carbon dioxide was evolved. After one crystallization from acetone, the residue was analytically pure. The acid was passed over the lead salt and then melted at  $78.5^{\circ}\text{C}$ . The yield of the isopentacosanic acid from isotricosyl iodide was 93 per cent of the theoretical.

0.6904 gm. substance required 18.00 cc. 0.1 N NaOH.

0.1000 " " : 0.2880 gm.  $\text{CO}_2$  and 0.1158 gm.  $\text{H}_2\text{O}$ .

$C_{26}H_{50}O_2$ . Calculated. C 78.53, H 13.09, Mol. wt. 382.

Found. " 78.54, " 12.96, " " 384.

*d-Cerebronic Acid*.—Two samples of cerebrosides were hydrolyzed for the preparation of *d*-cerebronic acid. Both were of the

less soluble material obtained on fractionation of the mixtures of crude cerebrosides isolated from brains and were practically pure phrenosin. Their rotations were determined in a solution of equal volumes of methyl alcohol and chloroform:

$$[\alpha]_D^{20} = \frac{+0.23^\circ \times 100}{0.5 \times 4.10} = +11.2^\circ$$

$$[\alpha]_D^{20} = \frac{+0.23^\circ \times 100}{0.5 \times 4.77} = +9.7^\circ$$

The cerebroside (500 gm.) was dissolved in 99.5 per cent alcohol (8,000 cc.) together with sulfuric acid (300 gm.) and the solution boiled for about 18 hours on the water bath. The ester crystallized at 0°C. It was recrystallized from acetone. The yield was 135 gm. It melted at 59.5°C. and had the following rotation in pyridine solution:

$$[\alpha]_D^{20} = \frac{+0.18^\circ \times 100}{1.0 \times 7.20} = +2.5^\circ$$

0.1003 gm. substance: 0.2793 gm. CO<sub>2</sub> and 0.1139 gm. H<sub>2</sub>O.

C<sub>27</sub>H<sub>54</sub>O<sub>8</sub>. Calculated. C 75.98, H 12.76.

Found. " 75.91, " 12.71.

The other cerebroside, hydrolyzed under the same conditions and worked up in the same manner, gave rise to an ester which melted at 60°C. and showed the following rotation:

$$[\alpha]_D^{20} = \frac{+0.25^\circ \times 100}{1.0 \times 5.56} = +4.5^\circ$$

0.1004 gm. substance: 0.2790 gm. CO<sub>2</sub> and 0.1165 gm. H<sub>2</sub>O.

C<sub>27</sub>H<sub>54</sub>O<sub>8</sub>. Calculated. C 75.98, H 12.76.

Found. " 75.78, " 12.99.

The cerebronic ester was saponified by boiling its alcoholic solution with a large excess of sodium hydroxide on the water bath over night. The alcohol was removed and the soap decomposed in the usual manner. The cerebronic acid was crystallized from acetone and passed over the lead salt. It was then analytically pure and melted at 99°C. Fractionation by means of the lithium salt did not separate any lignoceric acid. The rotation in pyridine was

$$[\alpha]_D^{20} = \frac{+0.29^\circ \times 100}{1.0 \times 8.33} = +3.5^\circ$$

0.7303 gm. substance required 18.27 cc. 0.1 N NaOH.

0.0997 " " : 0.2754 gm. CO<sub>2</sub> and 0.1106 gm. H<sub>2</sub>O.

C<sub>25</sub>H<sub>50</sub>O<sub>3</sub>. Calculated. C 75.38, H 12.56, Mol. wt. 398.

Found. " 75.33, " 12.42, " " 400.

The second cerebronic ester, subjected to the same procedure, gave rise to an acid which melted at 99.5°C. It was analytically pure and had the following rotation:

$$[\alpha]_D^{20} = \frac{+0.33^\circ \times 100}{1.0 \times 8.79} = +3.8^\circ$$

0.7346 gm. substance required 18.45 cc. 0.1 N NaOH.

0.1004 " " : 0.2787 gm. CO<sub>2</sub> and 0.1124 gm. H<sub>2</sub>O.

C<sub>25</sub>H<sub>50</sub>O<sub>3</sub>. Calculated. C 75.38, H 12.56, Mol. wt. 398.

Found. " 75.70, " 12.53, " " 398.

*Lignoceric Acid*.—The cerebronic acid (25 gm.) was oxidized in acetone solution. The procedure followed exactly that described for the oxidation of  $\alpha$ -hydroxylignoceric acid to isotricosan acid. The yield was 81 per cent of the theoretical. It was passed over the lithium and lead salts and then melted at 80.5°C.

0.7227 gm. substance required 19.42 cc. 0.1 N NaOH.

0.0999 " " : 0.2863 gm. CO<sub>2</sub> and 0.1166 gm. H<sub>2</sub>O.

C<sub>24</sub>H<sub>48</sub>O<sub>2</sub>. Calculated. C 78.26, H 13.04, Mol. wt. 368.

Found. " 78.15, " 13.06, " " 372.

The following descriptions represent two series of experiments. In one the starting point was the lignoceric acid from cerebronic acid, described above. In the second, the lignoceric acid from peanut oil, described at the beginning of the experimental part, served as the starting point. In each experiment one melting point is given. The mixed melting points are tabulated at the end of the introduction. Analytical data are given for both series; those of the members of the cerebroside series under (A) and of the others under (B).

*Ethyl Lignocerate*.—A solution of lignoceric acid (30 gm.) and sulfuric acid (10 gm.) in 99.5 per cent alcohol (500 cc.) was boiled over night on the water bath. The ester separated at 0°C.

Crystallization from 99.5 per cent alcohol gave an analytically pure product. The yield was 96 per cent of the theoretical. The ester melted at 55°C. as given in the literature.

(A) 0.1001 gm. substance: 0.2888 gm.  $\text{CO}_2$  and 0.1166 gm.  $\text{H}_2\text{O}$ .

(B) 0.1006 " " : 0.2894 " " " 0.1182 " "

$\text{C}_{26}\text{H}_{52}\text{O}_2$ . Calculated. C 78.79, H 13.13.

(A) Found. " 78.68, " 13.02.

(B) " " 78.45, " 13.15.

*Lignoceryl Alcohol*.—Ethyl lignocerate (10 gm.) was reduced with sodium and absolute alcohol. The process has already been described. Two crystallizations from acetone sufficed to give an analytically pure substance. 83 per cent of the ester was recovered as the alcohol and practically all of the remainder as lignoceric acid. The alcohol melted at 72°C. as given in the literature.

(A) 0.1006 gm. substance: 0.2990 gm.  $\text{CO}_2$  and 0.1268 gm.  $\text{H}_2\text{O}$ .

(B) 0.1004 " " : 0.2987 " " " 0.1262 " "

$\text{C}_{24}\text{H}_{50}\text{O}$ . Calculated. C 81.36, H 14.12.

(A) Found. " 81.05, " 14.11.

(B) " " 81.13, " 14.06.

*Lignoceryl Iodide*.—The alcohol (18 gm.) was heated with red phosphorus and iodine at 180°C. for 1 hour. The product was twice crystallized from acetone. It then melted at 48°C., as given in the literature, and was analytically pure. The yield was 96 per cent of the theoretical.

(A) 0.2010 gm. substance: 0.1024 gm.  $\text{AgI}$  (Carius).

(B) 0.2014 " " : 0.1032 " " "

$\text{C}_{24}\text{H}_{49}\text{I}$ . Calculated. I 27.37.

(A) Found. " 27.53.

(B) " " 27.69.

*Lignoceryl Cyanide*.—Lignoceryl iodide (10 gm.) was dissolved in 99.5 per cent alcohol (300 cc.) and finely pulverized potassium cyanide (11.5 gm.) added. The solution was boiled on the water bath for about 17 hours. The nitrile which separated at 0°C., was crystallized from acetone. It melted at 56.5°C.

(A) 0.2000 gm. substance required 5.20 cc. 0.1 N  $\text{HCl}$ .

(B) 0.2000 " " " 5.00 " 0.1 N "

$\text{C}_{25}\text{H}_{49}\text{N}$ . Calculated. N 3.86.

(A) Found. " 3.64.

(B) " " 3.50.



For the preparation of isopentacosanic acid the nitrile was not isolated.

*Isopentacosanic Acid*.—All of the ether-soluble material from the preparation of the cyanide was dissolved in 99.5 per cent alcohol with a large excess of sodium hydroxide. The solution was boiled on the water bath for 24 hours. The alcohol was then evaporated, the residue extracted with boiling acetone, and the insoluble soap decomposed with dilute hydrochloric acid on the water bath. The product was analytically pure after one crystallization from acetone. The yield of isopentacosanic acid from the lignoceryl iodide was 93 per cent of the theoretical. It melted at 78.5°C.

(A)	0.7621 gm. substance	required 19.80 cc. 0.1 N NaOH.
(B)	0.7839 " "	" 20.30 " 0.1 N "
(A)	0.1002 " "	: 0.2882 gm. CO <sub>2</sub> and 0.1168 gm. H <sub>2</sub> O.
(B)	0.1004 " "	: 0.2896 " " " 0.1159 " "
	C <sub>25</sub> H <sub>50</sub> O <sub>2</sub> . Calculated. C 78.54, H 13.09, Mol. wt. 382.	
(A)	Found.	" 78.43, " 13.04, " 385.
(B)	"	" 78.66, " 12.91, " 386.

*α-Bromoisopentacosanic Acid*.—Isopentacosanic acid (7.0 gm.) was melted on the water bath with red phosphorus (0.4 gm.). Bromine (11.0 gm.) was then slowly introduced and the mixture heated for 4 hours. An excess of bromine was present at the end. The acid bromide was decomposed, the acid dried and crystallized from 40–50° gasoline. The yield of pure acid was 95 per cent of the theoretical. It melted at 70°C.

(A)	0.2024 gm. substance:	0.0808 gm. AgBr (Carius).
(B)	0.1980 " "	: 0.0800 " " "
	C <sub>25</sub> H <sub>49</sub> O <sub>2</sub> Br. Calculated. Br 17.32.	
(A)	Found.	" 16.99.
(B)	"	" 17.20.

*dl-Cerebronic Acid*.—*α*-Bromoisopentacosanic acid (50 gm.) was suspended in 12 per cent aqueous sodium hydroxide (200 cc.) and heated on the water bath for 40 hours with violent stirring. The soap was then decomposed with dilute hydrochloric acid on the water bath and crystallized from acetone. After the acid had been twice crystallized from chloroform and passed over the lead salt, it melted at 92.5°C. The yield of analytically pure material

was 93 per cent. The yield of *dl*-acid from the naturally occurring *d*-cerebronic acid was approximately 60 per cent.

(A)	0.8090 gm. substance	required 20.35 cc. 0.1 N NaOH.
(B)	0.9150 " "	" " 22.81 " 0.1 N "
(A)	0.1004 " "	: 0.2782 gm. CO <sub>2</sub> and 0.1107 gm. H <sub>2</sub> O.
(B)	0.1004 " "	: 0.2784 " " " 0.1154 " "
	C <sub>25</sub> H <sub>50</sub> O <sub>8</sub> .	Calculated. C 75.38, H 12.56, Mol. wt. 398.
(A)	Found.	" 75.56, " 12.34, " " 398.
(B)	"	" 75.61, " 12.86, " " 401.5.

*Ethyl Isopentacosanate*.—Isopentacosanic acid (15 gm.) was dissolved in 99.5 per cent alcohol (150 cc.) together with sulfuric acid (5 gm.) and the solution boiled on the water bath over night. The ester which separated at 0°C. was twice crystallized from 99.5 per cent alcohol. The yield of analytically pure ester was 98 per cent. It melted at 57°C.

(A)	0.1003 gm. substance:	0.2899 gm. CO <sub>2</sub> and 0.1158 gm. H <sub>2</sub> O.
(B)	0.1004 " "	: 0.2916 " " " 0.1212 " "
	C <sub>27</sub> H <sub>54</sub> O <sub>2</sub> .	Calculated. C 78.95, H 13.26.
(A)	Found.	" 78.82, " 12.92.
(B)	"	" 79.20, " 13.50.

*Isopentacosyl Alcohol*.—The ester was reduced by means of sodium and alcohol in the manner already described for isotricosyl alcohol. Two crystallizations from acetone sufficed to give an analytically pure product. The yield was 92 per cent. The alcohol melted at 75°C.

(A)	0.1001 gm. substance:	0.2992 gm. CO <sub>2</sub> and 0.1269 gm. H <sub>2</sub> O.
(B)	0.0998 " "	: 0.2987 " " " 0.1282 " "
	C <sub>25</sub> H <sub>52</sub> O.	Calculated. C 81.45, H 14.22.
(A)	Found.	" 81.51, " 14.19.
(B)	"	" 81.62, " 14.37.

*Isopentacosyl Iodide*.—The alcohol was heated at 180°C. for 1 hour with iodine and red phosphorus. After two crystallizations from acetone, it was analytically pure and melted at 51.5°C. The yield was 98 per cent.

(A)	0.2026 gm. substance:	0.1002 gm. AgI (Carius).
(B)	0.2088 " "	: 0.1018 " " "
	C <sub>25</sub> H <sub>51</sub> I.	Calculated. I 26.53.
(A)	Found.	" 26.73.
(B)	"	" 26.34.

*Isopentacosane*.—Isopentacosyl iodide (8 gm.) was dissolved in hot glacial acetic acid (300 cc.) and the solution saturated with dry hydrogen chloride. Zinc dust was then added in small portions at intervals to maintain a vigorous evolution of hydrogen. The reduction was continued for 48 hours. The product was poured into water and the hydrocarbon filtered off. It was analytically pure after two crystallizations from 99.5 per cent alcohol. It then melted at 54°C. Crystallization from ether finally raised the melting point to 56°C. The yield of hydrocarbon from *d*-cerebronic acid was about 60 per cent of the theoretical.

(A) 0.1010 gm. substance: 0.3153 gm. CO<sub>2</sub> and 0.1329 gm. H<sub>2</sub>O.

(B) 0.1002 " " : 0.3126 " " " 0.1324 " "

C<sub>25</sub>H<sub>52</sub>. Calculated. C 85.13, H 14.87.

(A) Found. " 85.13, " 14.72.

(B) " " 85.08, " 14.79.

## THE ANTISCORBUTIC VITAMINE.

### I. A STUDY OF ITS SOLUBILITY FROM DESICCATED ORANGE JUICE.\*

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Very little work has been done upon the solubility of the anti-scorbutic vitamine. It is classed as a water-soluble vitamine. The evidence for this rests upon its solubility in a diluted and filtered orange juice and its presence in the water extract of fresh plant tissue. Whether it would be soluble in water in the absence of other compounds occurring with it in such materials as orange juice, fresh green tissue, etc., has not so far as we are aware been determined. Hess and Unger<sup>1</sup> have reported that it is soluble in 80 per cent alcohol.

Progress in an elucidation of the nature of this substance will come only as its chemical and physical properties are determined. While its instability to heat and ready destruction by oxidizing agents are now well known,<sup>2</sup> its solubility, destruction by specific fermentations, precipitability, etc., have not been studied.

In this paper data are presented on its solubility in a number of organic solvents, including butyl alcohol, methyl alcohol, various concentrations of ethyl alcohol, chloroform, ether, ethyl acetate, acetone, petroleum ether, and benzene. Various types of solvents were purposely included in this survey on solubility in order that advantage could be taken of the well known variations in solvent properties with difference in composition.

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

<sup>1</sup> Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1918, xxxv, 479.

<sup>2</sup> For references to literature on these points, see Ellis, N. R., Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, 1921, xlvi, 367.

## EXPERIMENTAL.

In our first experiments the orange juice was squeezed from fresh oranges, pressed through cheese-cloth, and 300 cc. were poured onto sheets of filter paper and dried before a fan at room temperature (about 20°C.). The paper was then broken up, placed in flasks, and subjected to the action of the solvent in portions of 200 to 300 cc. at room temperature for a period of 2 days. This was repeated twice—the solvent always being filtered after contact with the paper. The united extracts from the 300 cc. of desiccated orange juice were next evaporated on 1 kilo of our *scorbutic* ration which consisted of 69 parts of rolled oats, 25 parts of ground alfalfa hay (heated in the autoclave for 30 minutes at 15 pounds pressure), 5 parts of casein, and 1 part of common salt. Only 1 kilo of the ration was prepared at a time which was sufficient for four animals for about 10 days where there was normal consumption. This necessitated the preparation of a fresh ration about every 10 days. Guinea pigs weighing 200 to 400 gm. were used as the test animals with four animals in each lot. They were fed the scorbutic ration carrying the extracts of the orange juice from the time of initiation of the experiment.

In the first experiments we used:

1. 95 per cent alcohol, as purchased.
2. Acetone, (Merck preparation).
3. Benzene, (Merck preparation).
4. Petroleum ether, B. P. 60-90°.
5. Chloroform, (Merck U. S. P.)

Of the above solvents tried, the only demonstrable solution of the antiscorbutic vitamine was effected by the 95 per cent alcohol. All of the other solvents failed completely. The animals succumbed to scurvy in 5 to 7 weeks, although had they eaten daily 20 gm. of the ration (a conservative consumption for a normal guinea pig of 300 gm. weight) they would have received the equivalent of 6 cc. of desiccated orange juice.<sup>3</sup> In the case of the 95 per cent alcohol extract, four animals of weights varying from 250 to 400 gm. were used (Chart 1). They did not develop scurvy from

<sup>3</sup> The oranges used in this investigation were a gift by the California Fruit Growers Exchange, Los Angeles, California. We wish to express to them our appreciation of this courtesy.

the use of this ration for 16 weeks—establishing beyond question the solubility of this vitamine in 95 per cent alcohol.

The fact that 95 per cent alcohol was the only effective solvent among the several tried suggested not only the use of other solvents but a change in the plan of experimentation. It was possible that considerable destruction of the vitamine had occurred in the desiccation and other processes of manipulation, thereby so reducing the mass of vitamine left as to make it impossible to obtain an active extract, particularly when the solubility was of a lower order than that of the 95 per cent alcohol. Consequently, it was necessary to determine if this vitamine was still present in

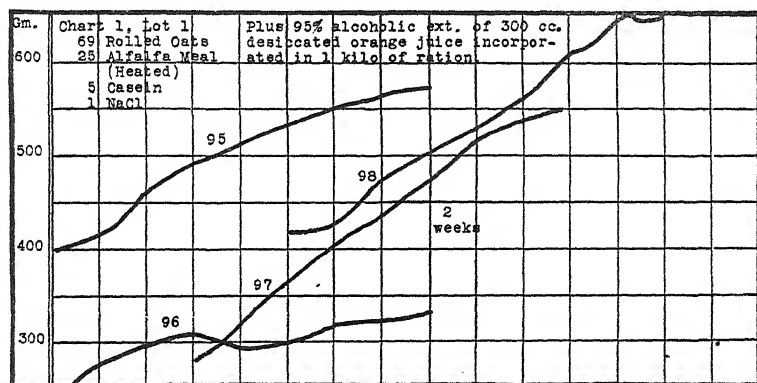


CHART 1. Illustrates the solubility of the antiscorbutic vitamine of desiccated orange juice in 95 per cent ethyl alcohol. Similar results were obtained with absolute and 80 per cent ethyl alcohol and with methyl alcohol.

the desiccated and extracted residues. To determine this the orange juice was evaporated by fan at room temperature on oatmeal, and this material then subjected to extraction by the particular solvent. Our plan was to desiccate 1,200 cc. of orange juice on 300 gm. of oatmeal first by the fan at room temperature (approximately 20°C.), and then in a large desiccator over calcium chloride for 6 to 10 days to remove moisture as completely as possible. This preparation was then extracted at room temperature with 2,000 cc. of the solvent which was left in contact with the material for 2 days; the solvent was filtered off by suction, the residue washed with the solvent, and this extraction repeated twice and in the same way.

The united extracts were evaporated at room temperature by the fan on 300 gm. of oatmeal, and in one case on the mixed ration. One-half of the 300 gm. of oatmeal, plus the evaporated extract, was used to make up 1,000 gm. of our basal ration (already described) and replaced in the ration an equivalent of the oatmeal. Had each animal eaten daily 20 gm. of this ration it would have received an equivalent of 12 cc. of desiccated orange juice. The residue from the extractions was also halved and mixed into enough of the ration to make 1 kilo, displacing an equivalent of oatmeal. By this procedure we not only tested the action of the solvent, but also the presence of the vitamine in the residue, thereby assuring ourselves that the vitamine had not been completely destroyed in the manipulations incident to the extracting and drying.

The solvents used included the following:

1. 95 per cent alcohol, as purchased.
2. 80 per cent alcohol, prepared by diluting the 95 per cent alcohol.
3. Absolute alcohol, prepared from 95 per cent alcohol by distillation from lime and metallic sodium (sp. gr. of preparation 0.7909 at 19°C.).
4. Acetone, (Merck preparation).
5. Benzene, (Merck preparation).
6. Petroleum ether, B. P. 60-90°.
7. Chloroform, (Merck U. S. P.)
8. Ethyl ether, anhydrous, alcohol-free, prepared by treating anesthesia ether with  $\text{CaCl}_2$  and metallic sodium and distilling.
9. Ethyl acetate, alcohol-free, prepared by treating high grade ethyl acetate with  $\text{CaCl}_2$  and distilling.
10. Methyl alcohol, aldehyde-free, prepared by treating with lime and distilling.
11. Butyl alcohol, absolute as purchased.

To determine the presence or absence of the vitamine in the extract or residue, we chose the method of feeding the scorbutic ration for about 2 weeks or until signs of scurvy, such as swollen wrists and crying when handled, made their appearance in the animals. When these symptoms appeared, the ration plus the evaporated extract was given to a group of four individuals and at the same time another group of four received the ration made from the extracted residue.

It will not be necessary to take the space required if charts of all the results were presented. These experiments showed that

the antiscorbutic vitamine of desiccated orange juice is insoluble in all of the solvents tried, with the exception of absolute, 95 and 80 per cent ethyl alcohol, and methyl alcohol. This confirms our

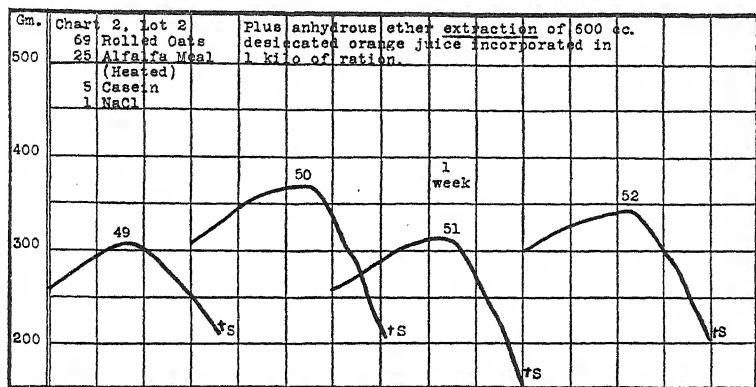


CHART 2. The insolubility of the antiscorbutic vitamine in anhydrous ethyl ether is shown in this chart. All the animals succumbed to scurvy.

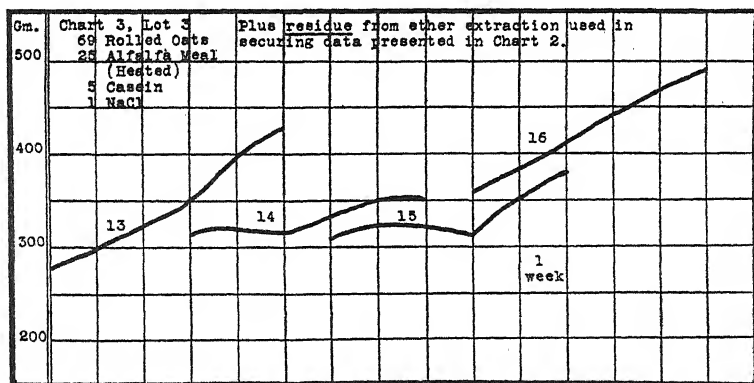


CHART 3. This chart records evidence that the antiscorbutic vitamine was left in the ether-extracted residue and had not been entirely destroyed in the manipulations of extraction.

first experiments as well as those of Hess and Unger. Further, it was found that while there was some destruction of this vitamine in the desiccating processes, nevertheless, the residue left after extraction still contained sufficient amounts of this substance to



induce recovery from scurvy and continued growth. Even in the case of the ethyl alcohol of varying concentrations not all of the vitamine was removed by the volume of solvent used, as shown in

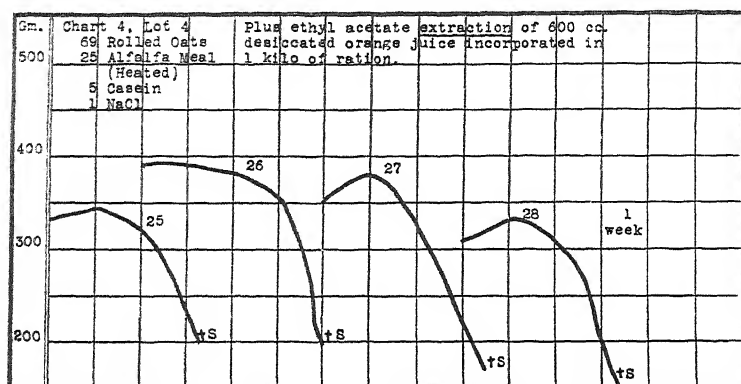


CHART 4. This chart illustrates the insolubility of vitamine C (antiscorbutic vitamine) in ethyl acetate. Similar results were secured with benzene, petroleum ether, acetone, chloroform, and butyl alcohol.

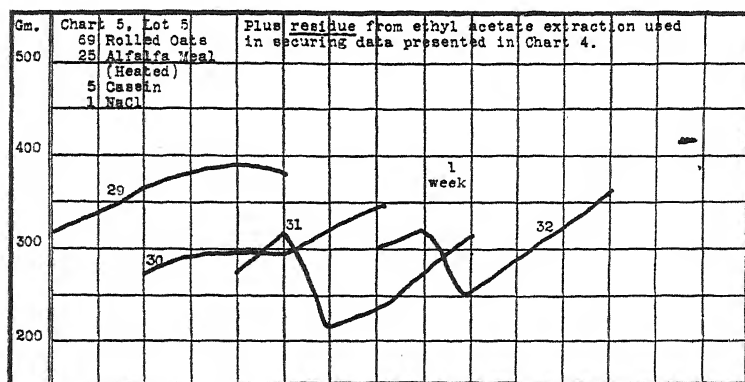


CHART 5. This chart illustrates the antiscorbutic potency of the ethyl acetate-extracted residue.

several experiments where the residue from the alcoholic extractions still showed antiscorbutic properties. This vitamine is more soluble in the 80 per cent alcohol than in the absolute alcohol, the residue from the extraction with the latter having distinctly potent

antiscorbutic properties. To illustrate the results typical of those materials that had no solvent action Charts 2, 3, 4, and 5 are introduced. Chart 2 shows the behavior of the animals with the *solvent* ether. Chart 3 shows the behavior on the *residue* from the ether extraction. Chart 4 shows the insolubility of the antiscorbutic vitamine in the *solvent* ethyl acetate. Chart 5 shows that the *residue* from the material extracted with ethyl acetate still contained sufficient antiscorbutic vitamine to protect against further progress of the disease.

The curves submitted for the behavior of ethyl ether and ethyl acetate are typical of all the results secured with those substances which had no solvent action. For ease of comparison, the data are summarized in Tables I and II.

TABLE I.

*Solubility of the Antiscorbutic Vitamine of Desiccated Orange Juice.*

Solvent.	Solubility.
Methyl alcohol.....	+
Ethyl alcohol, 95 per cent.....	+
“ “ 80 “ “ .....	+
“ “ absolute.....	+
Butyl “ .....	-
Acetone.....	-
Benzene.....	-
Petroleum ether.....	-
Chloroform.....	-
Ethyl ether.....	-
“ acetate.....	-

## SUMMARY.

1. The antiscorbutic vitamine of desiccated orange juice is soluble in 80 per cent, 95 per cent, and absolute ethyl alcohol. It is also soluble in methyl alcohol. The investigations of others show that it is also soluble in water, but these experiments do not exclude the influence on solubility of the salts inherent in the material extracted.

2. This vitamine of desiccated orange juice was found to be insoluble in butyl alcohol as well as in benzene, petroleum ether, acetone, ether, chloroform, and ethyl acetate.

3. The behavior of this vitamine toward organic solvents and water indicates that it is not of fat or lipid character.

TABLE II.  
*Successive Weekly Weighings of Guinea Pigs.*

Solvent.	Before the addition of the antiscorbutic.			After the addition of the antiscorbutic.*			
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Ether solution.		275	315	300	205	175	S+
		310	360	360	310	175	S+
		260	310	310	290	165	S+
		300	335	345	265	200	S+
Ether residue.	285	315	330	345	390	440	
	320	325	315	330	345	340	
	315	330	330	325	350	370	
	360	385	405	430	465	490	
Acetone solution.	245	295	315	280	200	185	S+
	210	260	285	205	165	S+	
	205	240	240	200	145	S+	
	245	285	265	195	160	S+	
Acetone residue.	275	310	320	335	345	380	
	240	280	280	305	325	315	
	315	360	380	445	370	390	
	195	225	240	270	285	320	
Ethyl acetate solution.	335	340	320	270	200	S+	
	390	385	360	335	370	195	S+
	350	375	335	295	220	180	S+
	310	335	320	250	190	155	S+
Ethyl acetate residue.	315	340		355	370	385	385
	275	295		290	295	320	320
	280	320		220	210	230	275
	300	315		260	280	290	335
Chloroform solution.	315	325		280	205	175	S+
	280	300		270	205	175	S+
	295	315		280	225	220	S+
	265	285		155	140	S+	
Chloroform residue.	280	295		178	+		
	255	275		265	235	265	310
	265	285		295	295	360	395
	265	285		240	275	315	360

\*S+ indicates scurvy and death.

TABLE II—*Continued.*

Solvent.	Before the addition of the antiscorbutic.			After the addition of the antiscorbutic.*			
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Benzene solution.	310	335		195	170	S+	
	275	305		225	160	S+	
	270	285		250	180	160	S+
	285	295		285	230	160	S+
Benzene residue.	355	390		385	225	180	+
	305	335		345	350	370	370
	370	390		410	440	495	510
	270	280		325	335	370	420
95 per cent alcohol solution.		315	330	375	390	390	350
		350	375	410	415	415	450
		315	335	355	310	350	350
		340	365	395	390	415	410
95 per cent alcohol residue.		340	355	360	360	360	385
		360	380	415	410	415	420
		285	300	300	270	275	260 S
		340	355	370	345	270	200 S+
80 per cent alcohol solution.	250	295	315	335	365	400	425
	260	300	310	310	335	370	375
	275	305	325	315	345	390	410
80 per cent alcohol residue.	250	280	275	240	150	S+	
	280	325	310	265	160	S+	
	285	315	315	275	205	S+	
	325	325	345	315	250	S+	
Absolute alcohol solution.		240	270	295	335	370	390
		250	270	275	285	315	325
		220	255	265	285	305	330
		245	265	285	305	335	345
Absolute alcohol residue.		195	230	260	290	315	355
		295	320	320	350	380	410
		235	235	240	275	315	360
		290	290	265	265	245	295

TABLE II—*Concluded.*

Solvent.	Before the addition of the antiscorbutic.			After the addition of the antiscorbutic.			
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Methyl alcohol solution.	365	385	400	375	235	+	
	355	375	390	400	458	500	500
	310	335	360	375	410	470	515
Methyl alcohol residue.		255	300	235	215	185	S+
		230	245	255	220	200	170
							S+
		210	240	225	230	185	145
							S+
		285	260	255	260	235	185
							S+
Petroleum ether solution.		330	295	255	S+		
		275	295	190	S+		
		225	240	200	185	150	S+
		275	265	205			
Petroleum ether residue.		300	290	225	+		
		275	280	215	255	295	310
		195	210	190	245	270	290
		310	350	370	410	430	430
Butyl alcohol solution.		270	270	255	160	S+	
		325	340	340	250	205	S+
		370	385	320	350	275	235
							S+
		265	275	245	185	175	S+
Butyl alcohol residue.		305	315	315	335	360	380
		280	290	310	335	365	380
		335	320	320	220	+	
		305	305	300	320	320	330

# DIGESTIBILITY OF RAW RICE, ARROWROOT, CANNA, CASSAVA, TARO, TREE-FERN, AND POTATO STARCHES.

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(From the Office of Home Economics, States Relations Service, United States Department of Agriculture, Washington.)

(Received for publication, February 23, 1922.)

## INTRODUCTION.

In tests previously reported<sup>1</sup> it was found that raw wheat and corn-starch, when eaten in quantity, were completely assimilated without any noted physiological disturbances and no starch was detected in the feces. Raw potato starch was much less completely digested, about one-fourth of the amount eaten being found in the feces on an average, and in many instances the subjects experienced pain or other physiological disturbances.

It seemed desirable to extend the work on the digestibility of raw starches to see whether complete digestibility was characteristic of other starches and to determine whether the less complete digestibility of potato starch (78.2 per cent on an average) was influenced by the amount eaten and also whether it was characteristic of the starch from other roots, tubers, and similar sources. The starches chosen were from rice, true arrowroot (*Maranta arundinacea*), so called commercial arrowroot (*Zamia floridana*), canna (*Canna edulis*), cassava (*Manihot utilissima*), taro root (*Caladium colocasia* or *Colocasia esculenta*), tree-fern (*Cibotium menziesii*), and potato, the last being ingested in smaller quantities than in the tests previously reported.

## EXPERIMENTAL.

The experimental procedure was the same as that followed not only in the previous work with raw starch but also throughout

<sup>1</sup> Langworthy, C. F., and Deuel, H. J., Jr., *J. Biol. Chem.*, 1920, xlii, 27.

the long series of digestion experiments made in this laboratory. The diet was so arranged that the foodstuffs under consideration was a predominating factor, the materials eaten with it being simple and with well understood characteristics. The foods were prepared in forms which experience had proved satisfactory for such experimental purposes. The separation of the feces, the analysis of foods and feces, the correction for metabolic products, and similar matters were accomplished by methods which had long been in use and the reliability of which had been carefully tested. The results of these experiments can therefore be directly compared one with another and with those of earlier ones.

In this connection, it is perhaps worth noting that investigators differ somewhat in their analytical and other procedures so that experiments reported from one laboratory may not be directly comparable with those from another laboratory where different methods are followed, and that such differences should be borne in mind in considering the findings of different investigators working with similar materials.

Such microscopic examination of the starches as were needed to determine the size and condition of the granules was made by Mr. G. L. Keenan of the Microchemical Laboratory, Bureau of Chemistry, United States Department of Agriculture. As in earlier experiments, each of the starches under consideration was eaten as the principal constituent of a frozen pudding of which the starch made up about 20 per cent. The pudding closely resembled ice-cream in texture and flavor and was made according to the following formula:

*Experimental Frozen Pudding.*

6 quarts milk.	2½ cups sugar.
4 pounds raw starch.	1 tablespoon salt.
3 cups table oil.	¼ cup lemon or vanilla extract.

The uncooked starch was mixed with the milk, sugar, and oil and immediately frozen in the same way as ice-cream. The flavoring extract masked to a great extent the taste of the uncooked starch and gave the frozen pudding a pleasing flavor.

That wetting and immediately freezing did not noticeably affect the starch granules was evident from a microscopic exami-

nation which showed that the grains were neither swollen nor broken.

The subjects were young men in normal health who had had experience in previous experiments and were thoroughly trustworthy. There was some variation in the groups in the different tests but this had no appreciable effect since the men were of much the same general type. With the exception of one of the authors (H. J. D.) and a soldier, the subjects were students in a local university. As in all the experiments in this laboratory the foods were attractive in appearance and the table service and other conditions were also good. The diet was not found monotonous during the 3 days (9 meals) which constituted each experimental period. There were intervals of not less than 4 days between experiments in which an ordinary mixed diet was followed. The subjects were given weighed portions of the raw starch frozen pudding along with a basal ration of oranges and sugar, and tea or coffee if desired.

On account of the number of experiments and extent of the protocols, the experimental data are somewhat condensed for the present report. Complete data are on file in the Office of Home Economics, United States Department of Agriculture.

#### *Rice Starch.*

Rice starch for these experiments was purchased from a commercial concern. The size of the granules was not measured, but it is known to be somewhat smaller than that of cassava starch.<sup>2</sup> Five experiments were conducted. The results are summarized in Table I.

The diet furnished on an average 25 gm. of protein, 52 gm. of fat, and 317 gm. of carbohydrate per man per day with an energy value of 1,830 calories. The average amount of raw starch eaten per man per day was 170 gm. No unusual physiological conditions were noted and the subjects remained in apparently normal health. The average coefficient of digestibility, 100 per cent, shows that the raw rice starch was completely digested.

<sup>2</sup> Thorpe, E., A dictionary of applied chemistry, London, New York, Bombay, and Calcutta, 2nd edition, 1913, v, 150.



TABLE I.

*Summary of Digestion Experiments with Raw Rice Starch in a Simple Mixed Diet.*

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of raw rice starch alone
		Protein.	Fat.	Carbohydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1,103	P. H. C.	84.2	94.9	98.8	82.6	100.0
1,104	W. V. D.	75.8	89.1	98.8	53.1	100.0
1,105	H. L. G.	84.3	94.1	98.9	71.0	100.0
1,106	E. L. M.	82.1	94.1	98.2	75.0	99.8
1,107	J. F. S.	89.2	96.7	99.3	87.9	100.0
Average .....		83.1	93.8	98.8	73.9	100.0

*True Arrowroot (Maranta arundinacea) Starch.*

*Maranta arundinacea* starch is the true arrowroot starch of commerce. That used in these experiments was obtained from the Hawaiian Agricultural Experiment Station. Microscopical examination of the starch granules showed them to be somewhat uniform in size. A large number of granules measured between 22 and 53 microns while the smaller granules which were much less numerous measured from 8 to 14 microns.

Two experiments were conducted with the *Maranta arundinacea* starch. The results are summarized in Table II.

TABLE II.

*Summary of Digestion Experiments with Raw True Arrowroot (Maranta arundinacea) Starch in a Simple Mixed Diet.*

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of raw arrowroot starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1,156	H. J. D.	60.2	95.1	94.4	74.4	92.1
1,157	E. L. M.	62.3	93.6	97.8	48.7	99.3
Average .....		61.2	94.4	96.1	61.6	95.7

The diet furnished on an average 17 gm. of protein, 56 gm. of fat, and 265 gm. of carbohydrate per man per day, with a fuel

value of 1,640 calories. The average consumption of raw starch was 124 gm. per man per day. No starch could be detected in the feces in Experiment 1,157 and so we may assume in that instance the starch was thoroughly digested. The feces in Experiment 1,156, however, gave a strong confirmatory test for starch. No data are available as to what the effect on the digestibility would be if the intake of raw arrowroot starch were increased to equal that of raw potato starch in previous work.<sup>1</sup>

*So Called Commercial Arrowroot (Zamia floridana) Starch.*

The starch used in these experiments was a commercial product obtained from Florida. The granules were somewhat larger than those from the *Maranta arundinacea*. An examination showed that three classes of starch granules were present: the larger grains varying from 70 to 42 microns; the medium from 20 to 14 microns; and the small granules from 8 to 1 microns. The large and medium-sized granules predominated.

Three experiments were made. The results are summarized in Table III.

TABLE III.

*Summary of Digestion Experiments with Raw Commercial Arrowroot (Zamia floridana) Starch in a Simple Mixed Diet.*

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of raw arrowroot starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1,125	H. L. G.	60.0	96.8	89.6	68.8	82.5
1,126	E. L. M.	47.2	92.0	77.8	60.0	65.7
1,127	J. F. S.	71.7	96.6	98.6	83.0	99.3
Average.....		59.6	95.1	88.7	70.6	82.5

The diet provided an average of 19 gm. of protein, 58 gm. of fat, and 297 gm. of carbohydrate per man per day and had a fuel value of 1,790 calories. The average amount of raw starch eaten per man per day was 168 gm. Subjects H. L. G. and E. L. M. remained in normal condition while J. F. S. noted a constipating effect ascribed to the diet. In Experiments 1,125 and 1,126 the quantity of feces voided was very large and contained much visible

unchanged starch. In the third experiment, however, the amount of feces voided was small and no starch could be detected by the iodine test.

As was noted with raw potato starch, there was considerable variation in the ability of the subjects to digest the raw starch. On account of the variation and the small number of experiments very little weight can be given to the average figure obtained, 82.5 per cent.

It is interesting to compare the results of the individual experiments in this series with those obtained from the earlier potato starch experiments with the same subjects. Subject H. L. G. digested 79 per cent of the raw potato starch as compared with 82 per cent of the *Zamia floridana*. Subject E. L. M. digested 62 per cent of the raw potato starch which compares very closely with his 65 per cent utilization of zamia starch. Subject J. F. S. digested practically all the zamia starch while he utilized about 95 per cent of the raw potato starch.

#### *Canna (Canna edulis) Starch.*

The starch used in these experiments was obtained through the courtesy of the Hawaiian Agricultural Experiment Station. Canna starch has the distinction of containing the largest granules of any of the common food starches, and that used in these experiments was composed almost entirely of large granules measuring from 95 to 42 microns. Although the potato starch which was used in the earlier experiments had some granules measuring as much as 95 microns, the canna starch contained a higher proportion of large granules.

Three experiments were conducted. The results are summarized in Table IV.

The diet provided on an average 22 gm. of protein, 80 gm. of fat, and 248 gm. of carbohydrate per man per day with an energy value of 1,800 calories. The average quantity of raw starch eaten was 109 gm. per man per day, a somewhat lower amount than in the other experiments.

A considerably smaller proportion of the canna starch was digested than of the other starches. Subject H. J. D. utilized only 43 per cent which is the minimum figure for all of the raw starch experiments reported from this Office. Subjects

H. L. G. and E. L. M. utilized a somewhat larger proportion of the canna starch but less than either had utilized in any of the other raw starch experiments. The average value, 53.3 per cent, for the digestibility of the raw canna starch is a lower figure than was found for any other raw starch studied. The subjects experienced no physiological disturbances and remained in normal health during the experiments.

TABLE IV.

*Summary of Digestion Experiments with Canna Starch in a Simple Mixed Diet.*

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of canna starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1,159	H. J. D.	63.0	97.4	68.9	64.2	43.0
1,160	H. L. G.	51.6	96.6	80.6	47.8	56.5
1,161	E. L. M.	67.8	94.9	81.1	45.6	60.3
Average .....		60.8	96.3	76.9	52.5	53.3

*Cassava (Manihot utilisima) Starch.*

The cassava starch used in these experiments was obtained from a commercial company. No measurements were made of the granules but those of cassava starch are known to be considerably smaller than those of potato and arrowroot starches and somewhat smaller than wheat or maize starches.<sup>3</sup>

Three experiments were conducted. The results are summarized in Table V.

The diet provided on an average 18 gm. of protein, 48 gm. of fat, and 246 gm. of carbohydrate per man per day with an energy value of 1,490 calories. The average starch intake per man per day was 140 gm. No unusual physiological conditions were noted and the subjects remained in normal health during the experimental period.

Although the digestibility of the raw starch after allowance was made for the undigested residues from the accessory foods

<sup>3</sup> Bailey, L. H., Standard cyclopedia of horticulture, New York, 1914-17, 3311.

was found to be only 98.8 per cent, no starch could be detected in the feces and it may be assumed that the raw starch was completely digested.

TABLE V.

*Summary of Digestion Experiments with Raw Cassava Starch in a Simple Mixed Diet.*

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of raw cassava starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1,118	P. H. C.	70.6	93.9	97.9	69.7	97.9
1,120	H. L. G.	65.5	92.8	97.4	63.0	98.4
1,122	J. F. S.	85.6	96.1	99.1	86.0	100.0
Average .....		73.9	94.3	98.1	72.9	98.8

*Taro root (Caladium colocasia or Colocasia esculenta) Starch.*

The taro root starch used in these experiments was obtained through the courtesy of the Hawaiian Agricultural Experiment Station. The starch is largely used in Hawaii in the cooked form of poi which, it is interesting to note, also frequently constitutes an important part in the diet of invalids. Bailey<sup>3</sup> states that in 1913 taro was the fourth crop in importance in the Hawaiian Islands. The granules of the taro root starch are extremely small, measuring between 1 and 7 microns.

Two experiments were conducted. The results are summarized in Table VI.

TABLE VI.

*Summary of Digestion Experiments with Raw Taro Root Starch in a Simple Mixed Diet.*

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of raw taro root starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1,164	H. J. D.	57.9	93.4	96.5	56.3	98.1
1,165	H. L. G.	66.9	96.6	97.0	63.6	99.5
Average .....		62.4	95.0	96.8	60.0	98.8

The diet provided on an average 19 gm. of protein, 47 gm. of fat, and 271 gm. of carbohydrate per man per day with an energy value of 1,580 calories. The average intake of raw taro root starch per man per day was 120 gm.

The results show that the raw starch was practically completely digested in both experiments. This is confirmed by the fact that no starch could be detected in the feces in either case.

*Tree-Fern (Cibotium menziesii) Starch.*

The tree-fern starch, obtained from the Hawaiian Agricultural Experiment Station, was composed of grains of varying size. The smaller grains, which measured about 9 microns, were apparently more numerous than the larger ones which measured about 27 microns.

Two experiments were conducted. The results are summarized in Table VII.

TABLE VII.

*Summary of Digestion Experiments with Raw Tree-Fern Starch in a Simple Mixed Diet.*

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of raw tree-fern starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1,162	H. L. G.	64.5	95.6	93.2	65.7	90.2
1,163	E. L. M.	73.8	96.6	96.6	67.1	96.5
Average .....		69.2	96.1	94.9	66.4	93.4

The diet provided on an average 23 gm. of protein, 71 gm. of fat, and 314 gm. of carbohydrate per man per day with an energy value of 1,990 calories. The average amount of raw tree-fern starch eaten per man per day was 150 gm.

Subject H. L. G. digested approximately 90 per cent of the raw starch and Subject E. L. M. about 96 per cent. When samples of feces were tested with iodine no distinct blue color was noted, but a brown color only. It would seem, therefore, that the proportion of undigested starch was very small.

*Potato Starch.*

In order to find out what effect a smaller ingestion of raw potato starch than that previously tested would have on its digestibility, experiments were conducted using a pudding made in the same way as before except that only one-half the quantity of starch was used.

Two experiments were conducted. The results are summarized in Table VIII.

TABLE VIII.

*Summary of Digestion Experiments with Raw Potato Starch in a Simple Mixed Diet.*

Experiment No.	Subject.	Digestibility of entire ration.				Estimated digestibility of raw potato starch alone.
		Protein.	Fat.	Carbohydrate.	Ash.	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1,139	C. J. G.	75.7	93.9	96.7	84.8	99.7
1,141	E. L. M.	67.4	92.3	95.8	73.9	94.5
Average.....		71.6	93.1	96.2	79.4	97.1

The diet provided on an average 17 gm. of protein, 40 gm. of fat, and 212 gm. of carbohydrate per man per day with an energy value of 1,280 calories. The average amount of starch eaten was 59 gm. per man per day which is somewhat less than one-third the amount which was eaten in the other potato starch experiments. There was practically complete utilization of the starch in the first experiment in which only 40 gm. per man per day were eaten, while in the second experiment in which approximately 70 gm. were eaten, there was a somewhat smaller coefficient of digestibility. In both cases the iodine test for starch in the feces was positive. The average digestibility for raw potato starch found in these two tests is 97.1 per cent and is considerably higher than the figure 78.2 per cent, previously obtained for potato starch eaten in about three times as great a quantity. It would seem from these tests that the smaller the quantity of potato starch eaten, the higher the coefficient of digestibility.

## DISCUSSION.

The experiments with raw starches conducted by the Office of Home Economics have demonstrated that certain raw starches, including corn, wheat, cassava, rice, and taro root were completely digested when eaten in amounts sometimes as large as 250 gm. a day. Raw tree-fern and true arrowroot (*Maranta arundinacea*) starches were nearly completely digested but some starch was present in the feces. Raw commercial arrowroot (*Zamia floridana*) and potato starches showed considerably less complete digestion, large quantities being present in the feces. Raw canna starch was even less digestible, its coefficient being only about 50 per cent.

In these experiments there seems to be a direct relationship between the size of the starch granules and its digestibility. Whether this relationship is accidental or not has not been determined. One might readily suppose that the larger starch granule contained a thicker cellulose covering and consequently one more impervious to the digestive ferments than the smaller granule. The factor of surface area might also come into consideration since the larger the granule, the proportionately smaller surface area for being attacked.

In the case of the larger granuled starches there appears to be a much larger variation between the ability of different subjects to digest the raw starch than could be accounted for on the basis of experimental error. The large number of digestion experiments previously conducted by this Office have, with the possible exception of some experiments on bran,<sup>4</sup> demonstrated no greater variation in the ability of the digestive organs of different individuals than might be attributed to experimental error.

There are also indications that smaller quantities of a given starch might be more completely digested but additional experiments are necessary to prove this conclusively.

<sup>4</sup> Holmes, A. D., *U. S. Dept. Agric., Bull.* 751, 1919.





## A METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF LACTIC ACID.

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### INTRODUCTION.

For quantities of lactic acid greater than 10 or 20 mg. the titration method of von Fürth and Charnass (1) is available. For much smaller quantities, the only methods hitherto proposed have been colorimetric (2). With the disadvantages of colorimetric methods in mind, study was made of a titration method which gives satisfactory results with as little lactic acid as 0.2 mg.

Chemical methods for determining lactic acid are based upon the following (3) reaction:



It is not the purpose of this paper to review the literature dealing with these methods. It should be pointed out that the reaction has been carried out under two sets of conditions. In one case (4), dilute  $\text{KMnO}_4$  is added to the boiling acidified solution, and the resulting acetaldehyde distilled off and determined. In the other case (5), the reaction is carried out at a higher temperature in the presence of concentrated sulfuric acid. Under these circumstances, either the aldehyde or the carbon monoxide may serve as a measure of the lactic acid present.

Two questions arise in connection with these methods. First, as to the specificity of the reaction: It will be shown that many other substances—especially the other  $\alpha$ -hydroxy-acids—yield aldehydes or ketones when subjected to either process. The separation of lactic acid from such interfering substances is by no means easy. This question will be discussed further in connection with the application of the proposed method to biological material.

The second question is concerned with the quantitative aspect of the reaction. In the present paper, it will be shown that either process can be adapted to the determination of small quantities of lactic acid.

The features of the method here presented are: a modification of the Ripper aldehyde titration with bisulfite; and the application of aeration to the transference of aldehyde from the reaction mixture to a solution of sodium acid sulfite.

#### EXPERIMENTAL PART.

##### *Titration of Acetaldehyde.*

The method of Ripper (6) is based on the following reaction:



In slightly acid or neutral solutions, the reaction very rapidly proceeds practically quantitatively to the right; and the excess of bisulfite may be titrated without including that "bound" in the double compound. This method of titration gives concordant results with 0.1 and 0.01 N solutions; but when 0.001 N concentration is used, the results are erratic. This may be due in part to the instability of such dilute bisulfite. It is not due to the instability of the double compound. For, if after the end-point is reached in a titration of excess bisulfite, a small amount of sodium bicarbonate is added, the bound sulfite may then be titrated; and the results are theoretical. The figure so obtained is practically, if not quite, independent of the quantity of bisulfite originally used. These facts form the basis of the modified method.

##### *Preparation of Materials.*

*Acetaldehyde* was prepared by distilling paraldehyde with a small amount of dilute  $\text{H}_2\text{SO}_4$ . The vapor was led through a reflux condenser filled with water at about  $30^\circ$  and received in a flask of ice water. The product was redistilled in the same apparatus three times. In this manner a 20 per cent solution was obtained. Dilutions to 0.1, 0.01, and 0.001 N were made as needed.

*Sodium acid sulfite* was prepared by leading  $\text{SO}_2$  gas into a saturated  $\text{Na}_2\text{CO}_3$  solution as long as  $\text{CO}_2$  was given off. The

color becomes pale greenish yellow, and the odor of  $\text{SO}_2$  is obvious. Dilutions were made only as needed.

*Iodine* was prepared in the usual way. A stock 0.1 N solution was diluted to make 0.01 or 0.001 N as needed. All solutions contain 2 per cent KI. The solutions were frequently checked against 0.1 or 0.005 N  $\text{Na}_2\text{S}_2\text{O}_3$  containing a little NaOH, which, in turn, were checked against standard 0.1 N  $\text{KIO}_3/\text{HIO}_3$  (3.2496 gm. per liter).

*Method in Detail.*

The solution of aldehyde to be analyzed is added to a considerable excess of bisulfite, whose exact amount need not be known.

TABLE I.

Titration of acetaldehyde by the Ripper method and by the modified method. Results are given in terms of cc. of standardized iodine. Acetaldehyde solutions prepared by diluting an approximately 0.05 N solution.

Acetaldehyde.	Titer by Ripper method.	Titer by modified Ripper method.
10 cc. 0.05 N	8.05 cc. 0.1 N iodine.	7.93 cc. 0.1 N iodine.
10 " 0.05 N	8.03 " 0.1 N "	7.83 " 0.1 N "
10 " 0.005 N	8.09 " 0.01 N "	8.08 " 0.01 N "
10 " 0.005 N	8.10 " 0.01 N "	8.08 " 0.01 N "
20 " 0.0005 N	14.90 " 0.001 N "	16.25 " 0.001 N "
10 " 0.0005 N	8.42 " 0.001 N "	8.10 " 0.001 N "
5 " 0.0005 N	3.00 " 0.001 N "	4.05 " 0.001 N "

After 10 minutes, the excess of bisulfite is oxidized by adding 0.1 N iodine, and the end-point is adjusted to a definite blue to starch. Sufficient saturated sodium bicarbonate solution is then added to discharge the blue color. The bound sulfite can now be titrated with accurately standardized iodine. The end-point is determined by a blank control. It will be seen that 2 atoms of iodine correspond to 1 molecule of aldehyde. Three points deserve attention; if the first end-point is exceeded, no great inaccuracy is introduced, because of the very slow velocity of reaction (B) to the left. The second end-point, though perfectly clear-cut, is not permanent, owing to secondary reactions, such as the formation of iodoform. This side reaction is much more rapid if the alkalinity is too high; as may happen if the bicarbonate used con-

tains much carbonate, or if too much bicarbonate be used. The titration of the double compound is somewhat slow. The method appears to be satisfactory for dilute solutions as may be seen by comparison with the standard Ripper titration (Table I).

Among the advantages of the method may be mentioned the fact that a large excess of bisulfite may be used, thereby making possible the aeration procedure for catching acetaldehyde. Among the disadvantages must be mentioned errors introduced by volatile substances, such as phenols, which react with iodine in weakly alkaline solution. Both the modified method and the original method yield values for acetone which are always low. This is due to the fact that the hydrolytic dissociation constant for acetone sodium bisulfite (7) is relatively large ( $3 \times 10^{-3}$ ) as compared with that for aldehyde sodium bisulfite, ( $2 \times 10^{-6}$ ). It can be shown theoretically and by experiment that if a considerable excess of bisulfite is present, the fraction of acetone bound is constant for any given concentration of bisulfite and hydrogen ion concentration. The error introduced by the presence of acetone can therefore be determined after removal of the aldehyde by Shaffer's method (8) on an aliquot of the solution to be analyzed.

#### *Isolation of Acetaldehyde from Reaction Mixture.*

Owing to its great volatility, acetaldehyde is liable to be lost when distilled. It was found, however, that the method of aeration lends itself readily to the transfer of acetaldehyde from one solution to another. This can be understood from a consideration of the distribution coefficient. At  $100^{\circ}$ , the ratio of the concentration of aldehyde in air and in water is 1:60; at  $4^{\circ}$ , the ratio falls to 1:1,760. If a slow stream of air be led through a small volume of the aqueous solution in a tube heated to  $100^{\circ}$ , and then through a large volume of ice water in two tubes in series, the transfer may be nearly quantitative. In one experiment, a liter of air passing through 5 cc. of solution at  $100^{\circ}$  removed 99 per cent of the aldehyde, transferring 96 per cent to 20 cc. of ice water in the first receiver and 3 per cent to a like volume in the second. If an excess of bisulfite is present in the receivers the aldehyde is so rapidly combined that subsequent losses do not occur, even if very large volumes of air pass. An ordinary aspiration pump is

used. No cooling of the receivers is necessary. In all the experiments to be reported, the receivers consist of  $20 \times 200$  mm. Pyrex tubes; the aeration tubes are of glass drawn to thick-walled capillaries, so that air passes through the fluid in a stream of small bubbles. Rubber tubing does not appear to hold back more than traces of aldehyde. The tube leading from the warmed reaction vessel is made of glass 40 cm. long and serves as an air condenser. Although the first receiver becomes warm from condensed water vapor, the catches of aldehyde are good (see Table II).

TABLE II.

Analysis of lithium lactate solutions by the permanganate method. The largest part of the acetaldehyde is found in the first receiving tube.

0.1 N lithium lactate.	Yield of aldehyde in terms of 0.01 N I.		Total minus blank.	Theory.	Yield.	Lactic acid present.
	Receiver 1.	Receiver 2.				
cc.					<i>per cent</i>	<i>mg.</i>
0	0.05	0.00				
1	18.00	0.75	18.70	20.00	93.50	9.0
1	18.65	0.20	18.80	20.00	94.00	9.0
1	19.00	0.10	19.05	20.00	95.00	9.0
0.001 N lithium lactate.	Yield in terms of 0.001 N I.		Total minus blank.	Theory.	Yield.	Lactic acid present.
	Receiver 1.	Receiver 2.				
cc.					<i>per cent</i>	<i>mg.</i>
0	0.25	0.05				
2	3.68	0.16	3.58	4.00	89.50	0.18
2	3.57	0.22	3.59	4.00	89.8	0.18
2	3.58	0.25	3.63	4.00	90.8	0.18

#### *Determination of Lactic Acid in Pure Solutions.*

Two lactic acid standards are used. Both were prepared from a commercial brand of lactic acid. *i*-Lithium lactate was prepared by adding the calculated quantity of  $\text{Li}_2\text{CO}_3$  to 50 per cent lactic acid, filtering, concentrating, and recrystallizing three times. Owing to high solubility, the yield was small, and the product was probably not pure. It possesses the advantages of having no water of crystallization. For most of the experiments, *i*-zinc lactate was used. It was prepared by adding the calculated amount of zinc chloride in solution to lactic acid previously neu-

tralized with NaOH. The product was recrystallized four times. It was free of chloride and its ZnO content was 27.32 per cent; theoretical for Zn ( $C_3H_5O_3$ )  $3 H_2O$  is 27.36 per cent. A 0.1 N solution contains 14.88 gm. per liter.

The reaction is carried out in a Pyrex glass tube  $20 \times 200$  mm. A straight inlet tube drawn to a capillary extends nearly to the bottom. The outlet tube is joined directly to a 40 cm. air condenser which, in turn, leads to the two receiving tubes. A rapid current of air is maintained by an aspiration pump. Usually four sets of analyses are run simultaneously. Rubber stoppers are used.

TABLE III.

Lactate used corrected for error in pipettes.		I used corrected for blank and titer.	Theory.	Yield.	Lactic acid.
			cc.	per cent	mg.
2.0	cc. 0.001 N	3.95 cc. 0.001 N	4.00	98.8	0.18
4.0	" 0.001 N	7.85 " 0.001 N	8.00	98.2	0.36
8.0	" 0.001 N	16.00 " 0.001 N	16.00	100.0	0.72
2.0	" 0.005 N	19.80 " 0.005 N	20.00	99.0	0.9
4.0	" 0.005 N	40.00 " 0.005 N	40.00	100.0	1.8
8.0	" 0.005 N	80.10 " 0.005 N	80.00	100.1	3.6
0.980	" 0.05 N	9.86 " 0.01 N	9.82	100.5	4.5
1.995	" 0.05 N	19.32 " 0.01 N	19.95	96.9	9.0
3.990	" 0.05 N	39.10 " 0.01 N	39.90	98.0	18.0
9.950	" 0.05 N	97.0 " 0.01 N	99.50	97.5	45.0

Analysis of zinc lactate by heating with  $H_2SO_4$ . Receivers in cases of 0.05 N zinc lactate contained 20 cc. of 0.1 N  $NaHSO_3$ .

Two methods for carrying out the reaction were tried. The first is a micro adaptation of the von Fürth-Charnass procedure. The reaction tube, containing 5 or 10 cc. of 1 per cent  $H_2SO_4$  and the lactic acid to be estimated, is placed in a water bath heated to  $95^\circ$ ; the receivers each contain 20 cc. of 0.02 N  $NaHSO_3$ . While a fairly rapid air current is passing, 0.005 N  $KMnO_4$  is added through the inlet tube, drop by drop; only as rapidly as it is decolorized. Water is added to preserve the original volume. After about  $\frac{1}{2}$  hour, the permanganate no longer fades, and the reaction is complete. The air current is run for 10 minutes longer. The contents of the receiver are then transferred to a flask and the bound aldehyde is determined by the above described

titration. The yield (Table II) is about 92 per cent of the theoretical, a figure corresponding closely with that found by von Fürth and Charnass for larger quantities of lactic acid. A factor, 0.05, was consequently used to calculate mg. of lactic acid from cc. of 0.001 N I.

When larger quantities of lactic acid than 10 mg. are determined the results of this method are liable to be low. Attention was therefore directed to a second procedure, that of Meissner and Schneyer, for the formation of acetaldehyde from lactic acid. 50 per cent sulfuric acid is allowed to react at  $140^{\circ}$  with the solution to be analyzed. The volume of the reaction mixture varies from 5 to 10 cc.; the tube is heated in a fusible alloy bath to  $140^{\circ}$ . The air current should be rapid. After an initial lag of 10 minutes, the aldehyde catch steadily increases and reaches a theoretical amount in 50 minutes. An hour is allowed for the reaction. It will be seen from Table III that the results with pure lactate solutions are satisfactory for 0.18 to 45 mg. of lactic acid. The blank, although fairly large, is constant.

#### *Application to Biological Material.*

Before applying these methods to biological material, it seemed important to study their results with various known substances. Table IV shows the yield of a bisulfite-binding product in terms of cc. of N iodine for a gram-molecule of each substance tested. It is seen that  $\alpha$ -hydroxy-acids in general give considerable yields as does glucose; glyceric aldehyde, gluconic acid, pyruvic acid, smaller yields; citric acid under the conditions of the test, yields acetone, which binds bisulfite. Amino-acids give very small yields.  $\beta$ -Hydroxybutyric acid gives only a small yield, as do creatinine and uric acid. Charring occurred during the  $\text{H}_2\text{SO}_4$  digestion only in case of glucose and gluconic acid but never with pure  $\alpha$ -hydroxy-acids. Amino-acids and urea do not interfere.

Another source of error must be pointed out. Many substances react with acetaldehyde in the presence of acid. Among these substances are the phenols. 1 mg. of phenol added to 1 mg. of lactic acid decreased the yield of aldehyde by the  $\text{H}_2\text{SO}_4$  method by more than 50 per cent. Charring results in 50 per cent  $\text{H}_2\text{SO}_4$ , although neither pure phenol nor lactic acid are so charred.



It is evident that the methods are not specific for lactic acid but apply to other  $\alpha$ -hydroxy-acids. Certain other compounds may be regarded as interfering substances. The problem of

TABLE IV.

Yield of bisulfite-binding substance from various compounds treated according to proposed methods for lactic acid. Results expressed in terms of cc. of  $N$  I solution per mol of substance. Theoretical for a hydroxy-acid should be 2,000.

Substance analyzed.	$N$ I per mol.	
	KMnO <sub>4</sub> method.	H <sub>2</sub> SO <sub>4</sub> method.
	cc.	cc.
Alanine.....	0	4
$\alpha$ -Aminobutyric acid.....	32	0
Aspartic acid.....	0	0
$\beta$ -Hydroxybutyric acid (synthetic).....	100	35
Citric acid.....	360	151
Creatinine.....	100	50
Cystine.....	0	2
Gluconic acid.....	202	270
Glucose.....	1,000	1,080
Glyceric acid.....	16.5	95.6
Glycocoll.....	0	9
Glycollic acid.....	36	865
Hippuric ".....	0.0	40
Histidine dihydrochloride.....	0	9
Leucine.....	7	0
Malic acid.....	255	1,012
Oxalic ".....	0	0
Phenol.....	76	10
Phenylalanine.....	32	0
Pyruvic acid.....	7.5	7.6
Succinic ".....	0	0
Tartaric ".....	27	370
Urea.....	0	0
Uric acid.....	220	52

separating lactic acid from such substances is by no means easy. One of the methods most frequently used is that of ether extraction from an acid aqueous solution. Extraction from solid dehydrating media (9), such as Na<sub>2</sub>SO<sub>4</sub>, or such as plaster of Paris, has also been advocated. Other solvents, such as amyl or butyl

alcohol and ethyl acetate (10) have also been advocated, because the distribution coefficient (11) of lactic acid between these and aqueous solutions is more favorable to rapid extraction. These solvents, of relatively high boiling point, do not adapt themselves as readily to any automatic extractor as does ether. For this reason, the use of ether was adopted. The theory of continuous liquid extractors has been well presented by Pinnow (11), and needs very little comment. Practical points deserving emphasis are that the rapidity of extraction varies directly (a) with the distribution coefficient (ratio of concentrations of extractable substance in ether and in aqueous solution); (b) with the rate of flow of ether; and (c) inversely with the volume of solution to be extracted. The latter point has not received due attention; consequently, we find in the literature that very long periods of time, up to 60 hours, are sometimes necessary for "complete" extraction. Most chemists, impressed by the "salting out" action of ammonium sulfate, have added this salt to the solution to be extracted, thus raising the distribution coefficient.

As the method described above for lactic acid is applicable to very small quantities, such as those found in 2 or 3 cc. of urine, it seemed desirable to devise a liquid extractor designed for such volumes of fluid. Its construction and operation may be explained best by reference to Fig. 1. *K* is a 300 cc. Kjeldahl flask containing 50 cc. of ether, warmed in a water bath. *H* is a Hopkins condenser. *F* is a funnel made from a test-tube; its end should be slightly bent, and its opening about 0.5 mm. *E*, the extraction tube, blown from a test-tube, has a side opening *O* for ether outflow, and a small bulb at the lower end. The narrow lower portion should contain about 2 cc. in a column about 10 cm. deep. A copper wire, *W*, holds *E*, and is carried between a cork, *C*, and the flask, *K*. As ether condenses and enters *F*, it gradually displaces the fluid in the stem; which should be of sufficient length to give such a head of pressure as to carry a rapid stream of ether in drops through the liquid in *E*, until a steady outflow takes place through *O*. Soon after starting operation of the apparatus, bubbles of vapor may fill the stem of *F*, and prevent flow. In this case it is only necessary to make momentary the air pressure at the side tube of the Hopkins condenser. The obstructing bubbles of vapor then collapse. After flow has begun,

the apparatus works very smoothly. It is usually run with the water bath at 60–70°. Liquids to be extracted are saturated with ammonium sulfate. It is convenient in practice to add about 1.3 gm. of the crystals (from a small graduated test-tube) to 2 cc. of fluid in *E*.

In studying the performance of this apparatus, 2 cc. quantities of solutions of various acids were placed in *E* and saturated by addi-

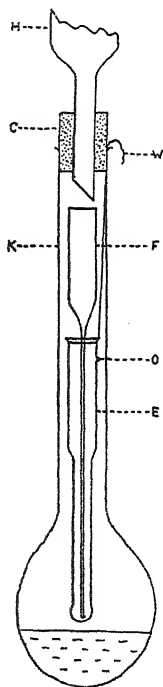


FIG. 1.

tion of 1.3 gm. of  $(\text{NH}_4)_2\text{SO}_4$ . From time to time, extraction was interrupted and the ether extract was titrated in the flask with 0.1 or 0.01 *N* NaOH, using phenolphthalein as indicator. The resulting figures were compared with the amount of acid known to be present at the outset, or with the total amount extractable. Results are tabulated in Table V.

It is evident that 99 per cent of lactic acid is extractable from 2 cc. of solution in  $\frac{1}{2}$  hour; that in this period, about 5 per cent of

citric acid, 60 per cent of glycollic acid, 100 per cent of  $\beta$ -hydroxybutyric acid, and 90 per cent of pyruvic acid are extractable. Only a trace of  $\text{H}_2\text{SO}_4$  is extractable; pyruvic acid (12) can be rendered unextractable by the presence of bisulfite; glucose is not extractable. It is thus possible to free lactic acid from some of the known substances that interfere with its estimation. It must be emphasized at this point, that biological fluids probably contain substances other than lactic acid which are extractable by ether and which yield bisulfite-binding compounds. Some of these, such as the other  $\alpha$ -hydroxy-acids, may even yield crystallized

TABLE V.

Extraction of various acids in  $\frac{1}{2}$  hour period, using continuous ether extractor. Results expressed in terms of 0.1 N acid.

Acid.	Taken.	Extracted.	Percentage extracted.
	cc.	cc.	per cent
Malic.....	1.8	0.3	16
Gluconic.....	2.2	0.05	2
Glyceric.....	12.52	2.0	16
Pyruvic.....	10.38	9.30	89.5
Glycollic.....	6.45	3.90	60.5
Tartaric.....	7.30	0.15	2
Citric.....	22.85	1.05	4.6
$\beta$ -Hydroxybutyric.....	20.0	20.0	100
Lactic.....	20.45	20.32	99.2

zinc compounds closely resembling zinc lactate. Moreover, phenols are extracted, and, as has been pointed out above, seriously lower the yield of acetaldehyde. One may, therefore, speak only provisionally of the total amount of ether extractable substances yielding aldehydes in terms of lactic acid. With this reservation in mind, the investigation was undertaken of the "lactic acid" content of urine and of blood.

#### *Determination of "Lactic Acid" in Urine.*

Since urine ordinarily contains very small amounts of lactic acid, and relatively large amounts of other carbon compounds, ether extraction was always employed before applying either the  $\text{KMnO}_4$  or the  $\text{H}_2\text{SO}_4$  method. It is quite certain that many other carbon compounds are thus extracted; such as phenols and fatty acids. This is evident from the fact that the ether extracts

reduce a very large amount of  $\text{KMnO}_4$  and char slightly when heated to  $150^\circ$  with  $\text{H}_2\text{SO}_4$ . These facts throw some doubt upon the figures obtained.

As a measure preliminary to ether extraction, it is necessary to remove albumin if present. This is accomplished by means of tungstic acid. To 5 cc. of urine are added 0.5 cc. of 10 per cent sodium tungstate, and 0.5 cc. of N sulfuric acid. After precipita-

TABLE VI.

Analysis of urine. Results expressed as mg. of lactic acid per 100 cc. and as lactic acid per kilo per 24 hours.

Case.	Diagnosis.	Lactic acid per 100 cc. of urine.	Lactic acid per kg. per day.	Blood lactic acid per 100 cc.
		mg.	mg.	mg.
1	Pneumonia, acute.....	38	?	32
2	Epilepsy (no recent attack).....	13	2.4	24
3	Cardiac decompensation .....	23	4.1	33
4	Normal (?).....	11	5.4	32
5	Bronchitis, chronic.....	11	4.4	25
6	Scabies.....	5	4.1	15
7	Pneumonia, convalescent.....	10	?	30
8	Chicken pox.....	11	2.3	44
9	Scarlet fever, convalescent.....	14	1.6	31
10	" " " .....	6	5.9	23
11	" " " .....	8.3	2.9	32
12	Athrepsia.....	6.5	6.4	
12	" .....	11.7	3.4	32.5
12	" .....	11.3	2.9	
13	" .....	19	4.2	
14	" .....	8	6.4	

tion, the urine is filtered, 2 cc. are placed in the extraction tube. It has been found possible to separate phenols from lactic acid by a very simple procedure. 2 cc. of albumin-free urine in the extraction tube are treated with a few drops of a strong phosphate buffer solution of pH 7.0, and extracted for 15 minutes. All the free phenols are then removed; 1.3 gm. of ammonium sulfate and a few drops of concentrated sulfuric acid are then added, and the lactic acid is extracted with a fresh supply of ether for  $\frac{1}{2}$  hour. To the ether are added about 10 cc. of water, 1 drop of 0.5 per cent phenolphthalein, and sufficient 0.1 N NaOH just to give an alkaline reaction. The ether is removed by distillation. The contents of the flask are transferred quantitatively to a small beaker,

and evaporated on a water bath nearly, but not quite, to dryness. The material is then washed with a small quantity of water into the reaction tube of the lactic acid apparatus, 10 cc. of 1:1  $\text{H}_2\text{SO}_4$  (by volume) or 5 cc. of concentrated  $\text{H}_2\text{SO}_4$  are added, and the determination is carried out as usual. Table VI shows figures for a few urines analyzed in this way. That lactic acid added to urine (containing albumin) may be recovered, is demonstrated both by direct titration of the ether extract and by the conversion to aldehyde. Blanks are fairly large, but constant. Although the ether contains large amounts of bisulfite-binding substances, these seem to be removed by the two evaporations. Acetone, preformed or split from acetoacetic acid, is also removed by evaporation. A source of error might arise in case of urines containing considerable  $\beta$ -hydroxybutyric acid. This error is greater if the  $\text{KMnO}_4$  method is used.

*Determination of "Lactic Acid" in Blood.*

The removal of protein from biological material deserves special note. Mondschein (13) has shown that under certain conditions lactic acid is not uniformly distributed between precipitated protein and filtrate. Van Slyke and Baker (14) call attention to the fact that casein has the power to adsorb considerable amounts of lactic acid and to the fact that at the isoelectric point of casein, such adsorption becomes negligibly small. Folin and Wu (15) find (empirically) that uric acid is adsorbed by protein precipitates, if the acidity of the medium is too high; the tungstic acid precipitation as elaborated by these workers avoids that difficulty. It also gives a filtrate satisfactory for the determination of lactic acid. This is indicated by the figures for recovery of lactic acid added to blood.

Recovery of lactic acid added to blood before precipitation of protein; 1 cc. of zinc lactate solution, diluted to correspond to final dilution of blood filtrate gave a liter of 51.2 cc. of 0.001 N I.

Zinc lactate.	0.001 N I.	Recovery of lactic acid.	Recovery.
cc.	cc.	cc.	per cent
0	16.8		
1	67.5	50.7	99.0
2	114.0	97.2	93.5

One of the most time-consuming steps in the usual methods for determination of lactic acid in blood has been the ether extraction. This is due, as has been indicated above, to the large volume of filtrate extracted. This difficulty can be obviated by evaporating the filtrates to a small volume. It must be remembered, however, that evaporation should be done under diminished pressure, in neutral or slightly acid solution, because at 100°, loss of lactic acid will occur if the solution is acid; and if the solution is alkaline, glucose readily yields compounds extractable by ether and yielding bisulfite-binding substances. In solutions too acid, glucose yields formic acid. It is likely that in blood filtrates the chief interfering substance from which lactic acid is separable by ether,

TABLE VII.

Comparison of lactic acid determinations in the same blood by the  $\text{KMnO}_4$  and the  $\text{H}_2\text{SO}_4$  method.

Case.	$\text{KMnO}_4$ per 100 cc.	$\text{H}_2\text{SO}_4$ per 100 cc.
	mg.	mg.
Normal child.....	23.0	21.0
Burn, child.....	42.8	33.1
" " .....	36.3	28.5
Rabbit 1.....	112.0	98.0
" 2.....	54.0	49.0
" 3.....	64.5	43.0

is glucose. Any method which would remove the glucose from solution would probably be satisfactory. A method proposed by Van Slyke (16) has been used for this purpose. To 10 cc. of the Folin-Wu filtrate, representing 1 cc. of blood, are added 2 cc. of 10 per cent  $\text{CuSO}_4$  and 2 cc. of 5 per cent suspension of  $\text{Ca}(\text{OH})_2$ . After being shaken at intervals for  $\frac{1}{2}$  hour the mixture is centrifuged, and 5 cc. duplicates of the filtrate are used for analysis. The blank in this case is determined by treating 10 cc. of 0.1 per cent glucose in the same way as the Folin-Wu filtrate. Results on ether extracts of evaporated blood filtrates agree well with those obtained on the copper hydroxide filtrate.

Blood lactic acid (in a case of cardiac failure):

Determination on ether extract of blood filtrate, *mg. per 100 cc.*

of blood..... 155

Determination by copper-lime method, *mg. per 100 cc. of blood.* 161

It is of interest to compare the results obtained by means of the  $\text{KMnO}_4$  method with results of the  $\text{H}_2\text{SO}_4$  method (Table VII). The results of the oxidation method tend to be slightly higher. Probably the  $\text{H}_2\text{SO}_4$  method gives results nearer the truth. It must be emphasized again that neither method is specific for lactic acid, but determines a group of substances. The same criticism

TABLE VIII.

Determinations of lactic acid in blood of convalescent children at rest in bed.

Case.	$\text{KMnO}_4$ method.	Case.	$\text{H}_2\text{SO}_4$ method.
	<i>mg. 100 cc.</i>		<i>mg. 100 cc.</i>
Empyema.....	25.9	Pneumonia.....	29.1
Nephritis.....	32.3	Epilepsy.....	24.0
Diphtheritic paralysis. ....	27.9	"Normal".....	32.1
Nephritis.....	35.6	Bronchitis.....	25.3
"Normal".....	21.8	Scabies.....	14.7
Cardiac.....	26.7	"Normal".....	30.7
Chorea.....	20.5	Convalescent scarlet fever.	31.2
".....	29.0	" " "	23.2
Typhoid.....	27.0	" " "	32.0
Harelip.....	25.4		
Tuberculous hip.....	28.0		
Pulmonary tuberculosis....	34.0		
Chorea.....	27.2		
Bronchitis.....	25.4		
Epilepsy.....	34.0		
Feeding.....	29.1		
Diabetes.....	28.0		
".....	20.0		
Maximum.....	35.6	Maximum.....	32.1
Minimum.....	20.5	Minimum.....	14.7
Average.....	28.1	Average.....	26.9

applies to methods proposed by others for lactic acid. The zinc salt (17), which is often isolated, weighed, and analyzed, frequently contains impurities, which may even prevent its perfect crystallization. If purified, loss inevitable occurs. Methods (18), depending upon measurement of the  $\text{CO}$  evolved when  $\text{H}_2\text{SO}_4$  acts upon lactic acid, are of less value because many other substances yield  $\text{CO}$  under like circumstances. Polariscopic methods (19) are



of value only when the presence of other optically active compounds can be excluded, and when large amounts of material are at hand.

It should be pointed out that pathological bloods containing acetone bodies (13) may yield too high results. In order to take account of the interfering action of acetone (preformed or derived from  $\beta$ -hydroxybutyric or acetoacetic acid), the method proposed by Shaffer and further studied by Hubbard was employed. After the final titration with 0.001 N iodine, the solution is transferred to a 300 cc. Kjeldahl flask, and about 1 gm. of sodium peroxide is added. The flask is joined to a condenser. In the receiver is placed 40 cc. of 0.02 N  $\text{NaHSO}_3$ . After heating the Kjeldahl flask 15 minutes in a boiling water bath, direct heat is applied and distillation is carried out for 4 or 5 minutes. It can be shown that acetaldehyde is completely oxidized, and that all the acetone may be recovered. After half an hour, the contents of the flask are titrated in the usual way. The blank thus obtained is subtracted from the original quantity of 0.001 N iodine. In normal cases it varies from 0 to 0.2 cc., and in most cases may be disregarded without great inaccuracy.

A summary is given of the findings in the blood of convalescent children (Table VIII).

#### SUMMARY.

1. A modification of the Ripper method for the titration of acetaldehyde is developed.
2. Aeration is applied to the transference of acetaldehyde from one solution to another.
3. The von Fürth-Charnass method for the estimation of lactic acid is adapted to quantities from 0.2 to 10 mg.
4. A method is developed, using 50 per cent sulfuric acid at  $140^\circ\text{C}$ ., for the estimation of from 0.2 to 45 mg. of lactic acid.
5. It is shown that methods such as these need special criticism when applied to biological material.
6. A method is elaborated for urine, which gives a provisional normal figure for the lactic acid content from 5 to 13 mg. per 100 cc.
7. A method is elaborated for blood, which gives a provisional normal figure from 15 to 32 mg. per 100 cc.

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## THE MINERAL METABOLISM OF THE MILCH COW.

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This investigation was suggested by the phenomenal mineral content of milk, and by observations on the behavior of milch cows which raised a question as to whether the mineral metabolism might not become a matter of critical importance in conditions of nutritional stress, especially as resulting from extreme development of the lactating function through selective breeding.

These observations were the following: (1) There is greater difficulty in getting a cow with calf after heavy and prolonged lactation than if bred comparatively soon after calving; (2) cows bred too young tend strongly to remain permanently small; (3) occasionally a cow will fail, unaccountably, after calving, to approach her normal milk production; (4) cows calving while in especially thin condition, or calving without having had an adequate dry, resting period, are apt to begin lactation at less than the normal rate, or, after a brief term of production, to fail abruptly; and (5) performance tests of milch cows, under conditions of forced production, have resulted in the loss of breeding capacity of so many superior cows as to occasion frequent comment and discussion among dairy cattle breeders.

That these unfavorable conditions have resulted from nutritive depletion seems unmistakable. To determine the importance of mineral metabolism in this relation was the primary object of this study, the method being to ascertain through a series of income and outgo experiments the rôle of the several mineral elements, and also nitrogen, in the annual cycle of lactation and gestation.

This investigation, involving the determination of 60 balances of intake against outgo with each of the elements sodium, potassium, calcium, magnesium, sulfur, phosphorus, chlorine, and ni-

trogen, as well as 12 balances of silicon and 6 of arsenic, comprises four studies, three of which have been published in detail, the fourth being the subject of this paper.

The first investigation<sup>1</sup> consisted of 18 balances of income and outgo, during liberal milk production, in the first half of the period of lactation. Normal rations of hay, grain, and silage, fed in quantities sufficient to maintain the live weight and to cause regular nitrogen and sulfur storage, led to invariable losses of calcium and magnesium from the body, and also negative balances of phosphorus in 15 out of 18 instances. The losses of calcium must certainly have involved drafts upon the mineral substance of the skeleton. The losses of these mineral elements which occurred during the feeding of timothy hay were larger than those occurring during the feeding of clover hay, but the cows were remarkably unresponsive to the large increase in calcium intake brought about by a change of roughage from timothy to clover hay. A considerable metabolism of silicon was demonstrated, especially as introduced into the ration by the timothy hay. This silicon was present in soluble compounds in amounts sufficient to render acid the normally alkaline urine.

The experiment, then, demonstrated the fact that the elaboration of milk in cows of good productive capacity, while on normal winter rations, involved drafts upon the mineral substance of the skeleton. In subsequent studies we have attempted to illuminate the conditions under which these mineral losses occur, and likewise the terms upon which these overdrafts are made good by storage.

In the second experiment,<sup>2</sup> we studied the effects of addition to the ration of large amounts of calcium carbonate, steamed bone flour, and sodium chloride, in a series of 12 balances. As in the first experiment, the subjects were in the first half of the period of lactation. Calcium was supplied in varying amounts up to double the maximum fed in the previous experiment.

As in the first study all calcium balances were negative. There was also loss of magnesium in all instances but one, but it was found to be possible for the cow to store phosphorus at the same

<sup>1</sup> Forbes, E. B., Beegle, F. M., Fritz, C. M., Morgan, L. E., and Rhue, S. N., *Ohio Agric. Exp. Station, Bull. 295*, 1916.

<sup>2</sup> Forbes, E. B., Beegle, F. M., Fritz, C. M., Morgan, L. E., and Rhue, S. N., *Ohio Agric. Exp. Station, Bull. 308*, 1917.

time that she was losing calcium. Excessive supply of calcium and magnesium then, failed to cause retention or even equilibrium of these elements; and the terms of the general mineral metabolism were not altered in important ways by doubling the intake of common salt.

In the third experiment,<sup>3</sup> comprising again 12 complete mineral balances, the subjects being, as before, in the first half of the period of lactation, the calcium content of some of the rations was still further increased, and a study was made of the influence upon the general mineral metabolism of three calcium compounds selected because of their ready solubility; namely, calcium lactate, calcium chloride, and precipitated bone phosphate.

The balances of calcium were, again, all negative; and there was no evidence that the limited utilization of calcium by lactating cows is due to difficult solubility of the calcium compounds of the ration, nor were any of the calcium compounds studied found to possess distinct superiorities over the others in their effects upon the calcium balance.

As in the previous studies the most salient points among the conclusions were the coincident *gains* in nitrogen (Period II) and *losses* of mineral nutrients, and, especially, a strikingly poor utilization of mineral supplements.

In spite of superabundant supplies of calcium, magnesium, and phosphorus in the rations the balances of calcium and phosphorus were in every case negative, and of magnesium 11 out of 12 balances were negative. This limited utilization of mineral nutrients was shown not to be due primarily to their conditions as to solubility.

Having accomplished a searching inquiry into the mineral metabolism of the milch cow during the period of most active production, and having demonstrated the existence of conditions during this earlier portion of the period of lactation which cannot possibly prevail during the entire productive life of the cow, it was our object in the fourth study especially to bring out the facts as to the course of the mineral metabolism through the whole of the annual cycle of lactation and gestation.

<sup>3</sup> Forbes, E. B., Halverson, J. O., and Morgan, L. E.. *Ohio Agric. Exp. Station, Bull. 330*, 1918, 111-118.

To accomplish this purpose the subjects of this experiment were so selected that the series of balance determinations would cover, in a disconnected way, the entire reproductive cycle, in recognition of the facts that the periodic variation in the mineral metabolism is a matter of critical importance, and that, both in relation to physiology and matters of practice, in milk production, the year is the unit.

Secondary objects were to determine the value of the particular mineral supplements used, including arsenic, and to continue our examination into the conditions of the general mineral metabolism of the cow, especially as to interrelationships between the elements.

The method of this study, as of the preceding three, involved the complete chemical accounting for feed, milk, urine, and feces. As in the earlier experiments six Holstein-Friesian cows were used, in three series of balance determinations, the collection periods being either 16 or 20 days in length, except as shorter periods were necessitated by the exigencies of the difficult experimental program. The collection periods were normally separated by 10 day intervals during which the subjects received the same ration which was to be used in the collection period to follow. The length of this intermediate period was extended in cases in which difficulty was experienced in getting the subjects onto a satisfactory basis of feed consumption and of general behavior.

The experimental subjects were all mature cows, in their prime (with one exception, to be noted), and were either high grades or pure-breds of first-class utility type. The cows in full milk varied from good to very good producers, as evidenced by their average daily milk yields of 37.87 to 61.36 pounds. All were in perfect health except No. 7, the history of which, subsequent to the completion of the experiment, indicates that she was not in normal condition during the investigation, but this fact seems to have had no important effects upon the results obtained in this study.

The cows were entirely dry in 7 balance determinations, and in 4 more were giving less than 10 pounds of milk per day. In the remaining 7 balance determinations the cows, as already stated, yielded from 37.87 to 61.36 pounds of milk per day.

In 11 out of 18 cases we were successful in keeping the cows in satisfactory condition for experimentation during the whole of the collection periods of 16 to 20 days as contemplated, but in the

remaining 7 cases the unusual difficulties of our experimental program required greater or less abbreviation of the collection periods. Results from the shorter periods are certainly of less value than those from the 16 to 20 day periods, but, even so, were mostly longer than the greater part of those reported in the literature.

These balance periods cover, with some interruptions, the entire cycle, from the first day of lactation to the day before parturition.

TABLE I.  
*Stage of Lactation and Gestation Covered by the Experiment.*

Period No.	Cow No.	Distinguishing feature of treatment.	Period of lactation.*	Period of gestation.	Average daily milk.
			<i>day</i>	<i>day</i>	<i>lbs.</i>
I	1	Calcium supplement.	139-158	49-68	42.77
	2	No supplement.	142-161	Not bred.	37.87
	3	Calcium supplement.	Dry.	246-265	Dry.
	4	No supplement.	"	240-259	"
	5	Calcium supplement.	55-74	Not bred.	40.63
	6	No supplement.	50-69	" "	49.61
II	3	No supplement.	1-7	Not bred.	47.48
	4	Calcium supplement.	Dry.	275-279	Dry.
	7	" "	Nearly dry.	226-229	9.79
	8	No supplement.	Dry.	248-263	Dry.
	9	Calcium supplement.	Nearly dry.	182-197	3.95
	10	No supplement.	" "	178-193	9.98
III	3	Arsenic; no calcium.	30-39	Not bred.	54.47
	4	" and "	24-33	" "	61.36
	7	" " "	Nearly dry.	250-265	1.28
	8	" no "	Dry.	273-279	Dry.
	9	" and "	"	209-221	"
	10	" no "	"	202-217	"

\* All days indicated are inclusive; that is, the period from the 49th to the 68th day includes both of these days, and is 20 days long.

The plan of this experiment is as indicated in Tables I and II, pages 285 and 286. In Table I the individuals are arranged in the three periods as fed; in Table II they are arranged in natural sequence, from beginning of lactation to end of gestation.

Table III, page 287, reports average daily amounts of feeds consumed, milk produced, and gain or loss in live weight; Table IV,



page 288, sets forth the composition of the milk, while the balance data are reported in Tables V, VI, and VII; pages 290 to 295.

The cows were selected in pairs at about the same stage in the period of lactation and gestation; and one of each pair was given a mineral supplement, while the other, for comparison, received the basal ration alone.

TABLE II.

*Enumeration of Individual Balance Periods, with Designation of Stages of Lactation and Gestation to Correspond, Arranged in Natural Sequence.*

Period No.	Cow No.	Distinguishing feature of treatment.	Period of lactation.	Period of gestation.	Average daily milk.
			<i>day</i>	<i>day</i>	<i>lbs.</i>
II	3	No supplement.	1-7	Not bred.	47.48
III	4	Arsenic and calcium.	24-33	" "	61.36
III	3	"	30-39	" "	54.47
I	6	No supplement.	50-69	" "	49.61
I	5	Calcium supplement.	55-74	" "	40.63
I	1	" "	139-158	49-68	42.77
I	2	No supplement.	142-161	Not bred.	37.87
II	10	" "	Nearly dry.	178-193	9.98
II	9	Calcium supplement.	" "	182-197	3.95
III	10	Arsenic.	Dry.	202-217	Dry.
III	9	" and calcium.	"	209-221	"
II	7	Calcium supplement.	Nearly dry.	226-229	9.79
I	4	No supplement.	Dry.	240-259	Dry.
II	8	" "	"	248-263	"
I	3	Calcium supplement.	"	246-265	"
III	7	Arsenic and calcium.	Nearly dry.	250-265	1.28
III	8	"	Dry.	273-279	Dry.
II	4	Calcium supplement.	"	275-279	"

The same mineral supplement was used in all cases, consisting of equal amounts of precipitated bone phosphate and precipitated calcium carbonate. The former is a by-product of gelatin manufacture, while the latter was a by-product of the manufacture of sodium hydroxide, in soap-making.

In the choice of this mineral supplement it was our design to supply calcium in readily soluble form, the precipitated bone phosphate being selected as cheap, readily accessible, and also a carrier of a useful acid element. The precipitated carbonate was

TABLE III.  
Average Daily Feeds Consumed, Milk Produced, and Gain or Loss in Live Weight.

Cow No.	Length of period.	Feeds consumed.							Milk produced.	Average daily live weight.	Average daily gain or loss in weight.
		Corn.	Cotton-seed meal.	Linseed-meal.	Wheat bran.	Alfalfa hay.	Sodium chloride.	Precipitated bone flour.	Precipitated calcium carbonate.		
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	cc.	gm.	kg.
Period I.											
1	20	5,161.0	397.0	397.0	397.0	7,720	44.452	54.9756	54.9756	19,400	-0.43
2	20	4,790.5	368.5	368.5	368.5	7,720	41.277			17,180	-0.89
3	20	2,947.8	226.8	226.8	226.8	5,448	25.401	61.5684	61.5684	588	+0.89
4	20	2,947.8	226.8	226.8	226.8	5,448	25.401			579	+0.17
5	20	4,790.5	368.5	368.5	368.5	7,720	41.277	47.1088	47.1088	18,430	+0.43
6	20	5,161.0	397.0	397.0	397.0	8,172	44.452			22,505	+0.33
Period II.											
3	7	2,951.0	227.0	227.0	227.0	5,448	25.424			21,537	-7.08*
4	5	2,951.0	227.0	227.0	227.0	5,448	25.424	62.7380	62.7380	594	-0.50
7	4	4,426.5	340.5	340.5	340.5	7,264	38.136	50.6160	50.6160	4,441	+0.83
8	16	2,951.0	227.0	227.0	227.0	5,448	25.424			465	+1.07
9	16	3,988.5	283.8	283.8	283.8	5,904	31.780	54.6568	54.6568	1,793	+1.65
10	16	4,426.5	340.5	340.5	340.5	7,264	38.136			4,525	+1.11
Period III.											
3	10	5,082.5	1,016.5		2,033.0	5,448	56.924	53.7000	53.7000	24,708	-0.53
4	10	5,082.5	1,016.5		2,033.0	7,264	56.924			27,833	-0.38
7	16	2,837.5	567.5		1,135.0	5,904	31.780	50.6160	50.6160	579	+1.44
8	7	2,270.0	454.0		908.0	5,448	25.424			493	+1.33
9	13	3,121.3	624.3		1,248.5	4,540	34.958	58.8000	58.8000	575	+1.20
10	16	3,405.0	681.0		1,362.0	5,904	38.136			546	+1.65

\* This extensive loss in weight occurred during the 7 days following parturition. Its extent is due largely to the fact that the ration was maintained the same in amount as in the previous period during which the cow was giving no milk.

TABLE IV.  
*Composition of Milk.*

Cow No.	Dry matter.	Ether extract.	Nitrogen.	Carbo-hydrate.	Ash.	Sodium.	Potassium.	Calcium.	Magnesium.	Sulfur.	Chlorine.	Phosphorus.
Period I.												
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	11.19	2.89	0.428	4.89	0.683	0.0363	0.1280	0.0887	0.0142	0.0269	0.0857	0.0772
2	11.49	3.10	0.452	4.77	0.741	0.0354	0.1264	0.1061	0.0128	0.0279	0.1109	0.0859
5	11.30	2.90	0.429	4.92	0.755	0.0457	0.1463	0.1035	0.0133	0.0262	0.0944	0.0917
6	11.30	3.22	0.411	4.71	0.747	0.0330	0.1633	0.1052	0.0121	0.0255	0.0907	0.0885
Period II.												
3	14.00	3.75	0.897	3.68	0.850	0.0706	0.1521	0.1247	0.0163	0.0575	0.1588	0.1146
7	11.23	3.03	0.638	3.30	0.830	0.1427	0.0755	0.0970	0.0157	0.0419	0.2118	0.0749
9	12.87	2.86	0.826	3.85	0.904	0.1431	0.0751	0.1727	0.0167	0.0532	0.2079	0.1164
10	13.29	3.37	0.639	5.08	0.771	0.0814	0.0919	0.1340	0.0164	0.0360	0.1209	0.1082
Period III.												
3	10.20	2.47	0.393	4.53	0.699	0.0408	0.1647	0.0924	0.0115	0.0232	0.1255	0.0794
4	11.47	3.00	0.447	4.92	0.706	0.0366	0.1509	0.1057	0.0139	0.0273	0.0884	0.0910
7	12.34	1.50	1.146	2.66	0.881	0.0239	0.0352	0.0643	0.0196	0.0753	0.2870	0.0701

added to the phosphate in order to supplement the calcium of the former, which, from a nutritional point of view, may be considered as present in deficient amounts as compared with phosphorus. The half-and-half proportion was determined arbitrarily. Of this mixture we fed a sufficient amount to supply, in each case, 70 mg. of calcium per kilo of live weight per day, based on live weights during preliminary or intermediate periods.

The basal ration in Periods I and II was composed of corn-meal 13 parts, and 1 part each of cottonseed meal, linseed-meal, and wheat bran, with alfalfa hay virtually *ad libitum*, and common salt in weights equal to 0.007 that of the grain.

It was found, however, that with alfalfa hay as the sole roughage this ration was more laxative than was desired. On this account the linseed-meal was eliminated in Period III, and the proportions of cottonseed meal and wheat bran were increased to make the the basal ration corn-meal 5 parts, cottonseed meal 1 part, and wheat bran 2 parts. These rations were of normal, standard type, but differed from the best in lacking corn silage.

The amounts of feed first offered the cows were determined by computation of their requirements. These amounts were then readjusted to meet the individual peculiarities of the subjects.

Arsenic in the form of Fowler's solution was fed to each of the six subjects, in Period III. The reasons for the inclusion of arsenic in this study were; (1) arsenic is being used as a component of certain commercial feeds for cows, (2) there is evidence in the literature in support of the idea that arsenic is effective to stimulate calcium retention, and (3) the public has an interest in knowing whether arsenic, administered to a cow, is eliminated to an appreciable extent in the milk.

The cows were fed and milked four times daily, at 6 hour intervals, and watered twice daily, after the morning and the evening feeding. They were weighed daily throughout the experiments. As usual the cows were watered with distilled water only, and the greatest care was used to guarantee the purity of this water. No water was placed in the storage tank without first testing it for contamination.

The cows were completely adapted to the routine before the beginning of the experiment, including the lights throughout the night, and there is no evidence that any of the peculiarities of the

TABLE V.  
Period I. Average Daily Balances of Minerals and Nitrogen.

Cow No.	Average daily milk yield.		Distinguishing features of rations.	Period of lactation and gestation.	Sodium.		Potassium.		Calcium.		Magnesium.		Sulfur.		Chlorine.		Phosphorus.		Nitrogen.	
	gm.	lbs.			Food.	Milk.	Food.	Milk.	Food.	Milk.	Food.	Milk.	Food.	Milk.	Food.	Milk.	Food.	Milk.	Food.	Milk.
1	19,400	42.77	Calcium supplement.	Lactation: Days 139-158.	Urine.	Feeces.	Urine.	Feeces.	Urine.	Feeces.	Urine.	Feeces.	Urine.	Feeces.	Urine.	Feeces.	Urine.	Feeces.	Urine.	Feeces.
				Gestation: Days 49-68.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.	Bal- ance.
2	17,180	37.87	No calcium supplement.	Lactation: Days 142-161.	22.2	137.2	129.6	32.7	29.2	39.2	32.7	29.2	29.2	39.2	32.7	29.2	32.7	29.2	39.2	32.7
				Gestation: Not bred.	7.0	24.8	17.2	2.8	5.2	16.6	2.8	5.2	10.7	5.8	0.1	113.4	15.0	83.0	113.4	15.0
3	Dry.		Calcium supplement.	Lactation: Days 240-265.	9.1	97.8	0.7	2.8	10.7	5.8	0.1	113.4	15.0	83.0	113.4	15.0	83.0	113.4	15.0	83.0
				Gestation: Days 240-265.	8.6	14.9	116.8	33.2	13.9	14.6	32.5	106.8	13.9	14.6	32.5	106.8	13.9	14.6	32.5	106.8
3	Dry.		Calcium supplement.	Lactation: Days 240-265.	-2.5	-0.3	-5.1	-6.1	-0.6	+2.2	-6.1	-0.6	-0.6	+2.2	+1.9	+0.2	+1.9	+0.2	+1.9	+0.2
				Gestation: Not bred.	20.9	135.1	93.5	31.3	28.3	35.9	31.3	28.3	28.3	35.9	31.3	28.3	35.9	31.3	28.3	35.9
3	Dry.		Calcium supplement.	Lactation: Days 240-265.	6.1	21.7	18.2	2.2	4.8	19.1	4.8	19.1	4.8	19.1	4.8	19.1	4.8	19.1	4.8	19.1
				Gestation: Not bred.	12.0	112.3	1.5	3.9	11.3	8.0	0.4	115.1	11.3	8.0	0.4	115.1	11.3	8.0	0.4	115.1
3	Dry.		Calcium supplement.	Lactation: Days 240-265.	4.7	9.3	83.9	30.6	13.7	8.4	23.8	107.2	13.7	8.4	23.8	107.2	13.7	8.4	23.8	107.2
				Gestation: Not bred.	-1.9	-8.2	-10.1	-5.4	-1.5	+0.4	-0.5	-5.7	-1.5	+0.4	-0.5	-5.7	-1.5	+0.4	-0.5	-5.7
3	Dry.		Calcium supplement.	Lactation: Days 240-265.	13.2	92.9	106.0	21.8	19.3	24.2	21.8	19.3	19.3	24.2	21.8	19.3	24.2	21.8	19.3	24.2
				Gestation: Not bred.	10.5	90.0	0.1	2.5	9.2	16.2	0.2	105.1	9.2	16.2	0.2	105.1	9.2	16.2	0.2	105.1
3	Dry.		Calcium supplement.	Lactation: Days 240-265.	3.3	4.7	98.9	21.5	8.7	6.9	28.8	59.0	8.7	6.9	28.8	59.0	8.7	6.9	28.8	59.0
				Gestation: Not bred.	-0.6	-1.8	+7.0	-2.2	+1.4	+1.0	+6.4	+33.2	+1.4	+1.0	+6.4	+33.2	+1.4	+1.0	+6.4	+33.2

4	Dry.	No calcium supplement.	Lactation: Dry. Gestation: Days 240-259.	13.2	92.9	65.8	21.1	19.1	22.8	25.1	197.2
				9.5	87.6	0.1	2.6	8.8	14.4	0.1	104.8
				3.3	4.6	59.1	21.5	8.5	4.8	18.9	61.8
				+0.4	+0.7	+6.6	-3.0	+1.8	+3.6	+6.1	+30.6
5	18,430	Calcium supplement.	Lactation: Days 55-74. Gestation: Not bred.	20.9	135.1	124.3	31.8	28.4	36.9	46.4	294.4
				8.4	27.0	19.1	2.5	4.8	17.4	16.9	79.0
				7.9	101.9	0.4	5.3	11.4	8.1	0.1	111.9
				5.7	11.9	143.0	33.8	14.4	11.4	39.9	108.8
				-1.1	-5.7	-38.2	-9.8	-2.2	+0.0	-10.5	-5.3
6	22,505	No calcium supplement.	Lactation: Days 50-69. Gestation: Not bred.	22.4	143.5	99.1	33.4	30.1	38.5	41.2	313.7
				7.4	36.8	23.7	2.7	5.7	20.4	19.9	92.5
				10.9	97.4	0.1	4.5	10.5	3.3	0.1	109.6
				5.3	10.9	83.1	31.6	13.9	11.7	23.1	112.0
				-1.2	-1.6	-7.8	-5.4	+0.0	+3.1	-1.9	-0.4

TABLE VI.  
Period II. Average Daily Balances of Minerals and Nitrogen.

Cow No.	Average daily milk yield.		Distinguishing features of rations.	Period of lactation and gestation.	Sodium.	Potassium.	Calcium.	Magnesium.	Sulfur.	Chlorine.	Phosphorus.	Nitrogen.
	gm.	lbs.			Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.
3	21,537	47.48	No calcium supplement.	Lactation: Days 1-7. Gestation: Not bred.	15.8	93.1	67.6	21.8	19.5	22.6	25.0	200.7
					15.2	32.8	26.9	3.5	12.4	34.2	24.7	193.1
					8.5	65.1	0.1	2.1	9.0	1.3	0.1	115.8
4	Dry.		Calcium supplement.	Lactation: Dry. Gestation: Days 275-279.	2.6	8.3	50.2	18.4	7.6	5.2	15.5	58.2
					-10.5	-13.1	-9.6	-2.2	-9.5	-18.1	-15.3	-166.4
					15.8	93.1	108.5	22.4	19.8	24.0	35.5	200.8
7	4,441	9.79	Calcium supplement.	Lactation: Nearly dry. Gestation: Days 226-229.	8.1	77.2	0.3	1.9	7.7	12.4	0.2	77.7
					3.6	6.0	94.2	20.2	8.1	5.3	27.1	63.4
					+4.1	+9.9	+14.0	+0.3	+4.0	+6.3	+8.2	+59.7
					22.8	126.9	123.4	30.6	27.2	34.1	44.1	279.8
					6.3	3.4	4.3	0.7	1.9	9.4	3.3	28.3
					4.9	116.5	0.3	5.1	9.8	14.1	0.2	99.1
					1.4	15.3	120.6	26.2	12.7	6.8	38.4	109.9
					+10.2	-8.3	-1.8	-1.4	+2.8	+3.8	+2.2	+42.5

8	Dry.	No calcium supplement.	Lactation: Dry. Gestation: Days 248-263.	15.8	93.1	67.6	21.8	19.5	22.6	25.0	200.7
				8.6	83.5	0.4	4.8	8.3	9.8	0.2	88.8
				4.4	14.5	59.0	17.0	8.1	8.0	19.4	62.9
				+2.8	-4.9	+8.2	-0.0	+3.1	+4.8	+5.4	+49.0
9	1,793	Calcium supplement.	Lactation: Nearly dry. Gestation: Days 182-197.	18.9	103.6	109.2	25.3	22.4	28.5	38.6	229.7
				2.6	1.3	3.1	0.3	1.0	3.7	2.1	14.8
				6.4	86.2	0.4	2.7	9.3	7.2	0.1	101.9
				4.2	14.5	97.5	23.2	9.5	13.2	31.2	75.6
				+5.7	+1.6	+8.2	-0.9	+2.6	+4.4	+5.2	+37.4
10	4,525	No calcium supplement.	Lactation: Nearly dry. Gestation: Days 178-193.	22.8	126.9	90.5	30.1	27.1	33.0	35.7	279.8
				3.7	4.2	6.1	0.7	1.6	5.5	4.9	28.9
				11.3	97.2	0.5	6.0	10.8	15.2	0.2	111.7
				3.1	10.2	75.4	25.5	11.2	8.8	25.2	87.0
				+4.7	+15.3	+8.5	-2.1	+3.5	+3.5	+5.4	+52.1



TABLE VII.  
Period III. Average Daily Balances of Minerals and of Nitrogen.

Cow No.	Average daily milk yield		Distinguishing features of rations.	Period of lactation and gestation.	Sodium.	Potassium.	Calcium.	Magnesium.	Sulfur.	Chlorine.	Phosphorus.	Arsenic.	Nitrogen.
	gm.	lbs.			Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.	Food. Milk. Urine. Feces. Bal- ance.
3	24,708	54.47	Arsenic, no calcium supplement.	Lactation: Days 30-39.	26.0 10.1	125.1 40.7	65.8 22.8	36.5 2.8	27.9 5.7	41.8 31.0	57.4 19.6	0.288 0.087	315.5 97.2
				Gestation: Not bred.	3.9 11.6 +0.4	63.3 23.8 -2.7	0.1 58.5 -15.6	5.8 31.5 -3.6	9.9 12.3 -0.0	2.6 6.0 +2.2	4.1 44.6 -10.9	0.154 0.047 +0.047	109.7 106.4 +2.2
4	27,833	61.36	Arsenic, calcium supplement.	Lactation: Days 24-33.	26.9 10.2	151.1 42.0	121.3 29.4	42.0 3.9	31.2 7.6	44.4 24.6	70.2 25.3	0.288 0.096	358.2 124.4
				Gestation: Not bred.	11.5 6.8 -1.6	100.8 12.5 -4.2	0.1 104.1 -12.3	2.9 36.3 -1.1	10.7 14.4 -1.5	7.7 13.5 -1.4	2.3 49.1 -6.5	0.141 0.051 +0.051	103.6 124.2 +6.0
7	579	1.28	Arsenic, calcium supplement.	Lactation: Nearly dry.	15.9 1.4	111.0 0.2	102.2 0.4	28.7 0.1	22.3 0.4	26.8 1.7	46.5 0.4	0.288 0.000	243.7 6.6
				Gestation: Days 250-265.	5.0 8.9 +0.6	88.9 18.9 +3.0	0.2 94.2 +7.4	5.9 21.9 +0.8	10.0 10.2 +1.7	11.0 14.9 -0.8	0.9 40.0 +5.2	0.083 0.137 +0.068	114.9 76.5 +45.7

8	Dry.	Arsenic, no calcium supplement.	Lactation: Dry. Gestation: Days 273-279.	13.1	99.2	63.6	24.5	19.4	21.2	32.1	0.288	212.0
				2.0	84.2	0.8	4.4	9.2	9.7	0.2	0.090	104.6
				10.3	22.3	57.1	23.2	11.9	5.3	43.7	0.102	101.3
				+0.8	-7.3	+5.7	-3.1	-1.7	+6.2	-11.8	+0.096	+6.1
9	Dry.	Arsenic, calcium supplement.	Lactation: Dry. Gestation: Days 209-221.	16.6	94.1	92.2	26.3	20.1	27.9	47.6	0.288	222.0
				8.4	87.0	0.1	4.4	10.3	15.5	0.1	0.074	124.7
				4.7	8.5	87.0	21.4	8.5	8.0	45.6	0.106	65.7
				+3.5	-1.4	+5.1	+0.5	+1.3	+4.4	+1.9	+0.108	+31.6
10	Dry.	Arsenic, no calcium supplement.	Lactation: Dry. Gestation: Days 202-217.	18.6	116.3	69.7	30.6	23.9	29.9	43.3	0.288	264.6
				10.5	106.6	0.3	5.0	13.0	21.5	0.2	0.056	147.8
				5.7	13.8	65.6	26.3	10.2	7.2	40.9	0.108	79.2
				+2.4	-4.1	+3.8	-0.7	+0.7	+1.2	+2.2	+0.124	+37.6

mineral metabolism were due to disturbance caused by the experimental procedures. A chemist was present at all times during the 24 hours, to guarantee correct weights, measures, and conditions of care; and the subjects were in a perfectly normal state of comfort and composure. Lack of exercise may conceivably have affected the mineral metabolism in specific ways, though we have no evidence or knowledge to warrant an assumption that this was true or likely to be true.

In 12 out of the 18 balances the cows gained in weight, and among the 7 balances in which the cows gave no milk the subjects gained in weight in 6 instances. The dry cows gained, on an average, 0.83 kilo, or 1.83 pounds, per head per day.

Among the milking cows the four which were nearly dry all gained in weight, while among the seven in full milk five lost and two gained in weight.

### *Discussion of Results.*

*Composition of the Milk.*—That the gross composition of the milk is determined by the age, breed, individuality, and state of health of the cow, and by the time in the period of lactation, and not in any definite way by the composition of the ration, is so thoroughly established and generally understood as to require no discussion. Certain of the variations in composition as related to the time elapsed since calving, however, are worthy of note.

Cow 3, Period II, was on experiment during the first 7 days of lactation. The milk from this period included the colostrum. The analysis shows that this milk, as compared with that from Cows 1 and 2, Period I, which between them covered the 139th to the 161st days of lactation, contained approximately twice the proportion of nitrogen, sodium, and sulfur, and a less pronounced excess of each of the other mineral elements. The laxative character of the colostrum may readily be due to the minerals which it holds in solution.

At the other end of the period of lactation stood Cow 7, Period III. This cow was milked only four times during the 16 day period. The secretion was curdy, and distinctly not normal milk. It was very low in fat, sodium, potassium, and calcium, and high in nitrogen, magnesium, sulfur, and chlorine. This milk also contained 6 parts per million of arsenic, but this fact is thought not

to be important in connection with normal milk secretion since this cow was not "giving milk" in the ordinary sense, and the secretion was not of usable character. The milk of the fresh cows (Nos. 3 and 4) contained no arsenic.

Cows 7, 9, and 10, Period II, were well along toward the end of the period of lactation. They produced from 4 to 10 pounds of milk per head per day. Their milk was normal and usable, but the analysis shows that it differed from milk produced at the middle of the period of lactation by being high in nitrogen and in all of the minerals except potassium, which was distinctly low. Sodium and chlorine were in much greater excess than the other mineral elements.

*Balance Determinations.*—Tables V, VI, and VII, pages 290 to 295, show: (1) the average daily milk yield, in grams, and in pounds; (2) the distinguishing features of the experimental feeding; (3) the status of the subjects, as to lactation and gestation; (4) the amounts of each of the mineral elements, and of nitrogen, in feed, milk, urine, and feces; and (5) the balance of income to outgo for each of these elements, eight in number in Periods I and II, and nine in Period III.

The subjects of this experiment having been selected in pairs representing the same stage in the annual cycle, one receiving a mineral supplement and the other the basal ration alone, in each case, we shall consider them in this light.

Cows 1 and 2, Period I, representing days of lactation 139 to 161, were not bred, and with milk production of 37.87 to 42.77 pounds, exhibited negative mineral balances, as a rule; and these together with the status of the sulfur and nitrogen balances, indicated a slightly insufficient food intake.

Cows 3 and 4, both dry, representing days of gestation 240 to 265, received feed enough to provide for retention in 12 out of the 16 individual elemental balances, including calcium, phosphorus, sulfur, chlorine, and nitrogen, for both cows. These positive balances are due to the cows having a sufficiency of feed, being dry, and being in the later part of the period of gestation.

Cows 5 and 6, in full milk, and not bred, days of lactation 50 to 74, like Cows 1 and 2 were slightly underfed, and, being also rather recently fresh, with the lactating impulse still at its height, and the milk secretion liberal (40.63 to 49.61 pounds), the balances were almost all negative.

After this period Cows 1, 2, 5, and 6 were discarded, Nos. 3 and 4 continued, and Cows 7, 8, 9, and 10 added.

Cows 3 and 4, Period II, were normally of about the same weight (Table III, Period I, page 287). In Period II, Cow 3 had just calved, and No. 4 was just about to calve. Their feed intake was the same, except that No. 4 was given a mineral supplement. The balances of these cows afford a striking demonstration of the essential character of metabolism at the beginning and at the end of the annual cycle.

Cow 4, during the last 5 days of pregnancy, was storing each of the mineral nutrients and nitrogen at a rapid rate, as shown by the 8 positive balances, while No. 3, immediately after parturition, on the same ration, except for the lack of the calcium supplement, lost largely of each of the elements studied. These losses were due in part to the liberation of a flood of nutrients, stored up during gestation, and in part to the insufficiency of the ration. It is interesting and significant that No. 4, just before calving, was storing calcium more rapidly than any other cow at any other period of the annual cycle. We believe that this result fairly represents a fact, that calcium, the balance of which is always negative during the early part of the period of lactation, is stored in abundance during gestation, especially during its last stages.

Cows 7 and 8, representing days 226 to 263 in the period of gestation, one being dry and the other nearly dry, were nearly ready to calve. The balances of sodium, sulfur, chlorine, phosphorus, and nitrogen were all positive, as also was the calcium balance in the case of the dry cow, which was also nearest to calving.

These balances indicate, as in the cases of Cows 3 and 4 in Period II, a sufficiency of feed, and the later and more rapid storage period in the term of gestation.

Cows 9 and 10 were not quite so far along in gestation, but told the same story, of spent impulse to lactate, and of storage in anticipation of parturition, all balances except magnesium being positive. Under these circumstances it seems impossible that the negative magnesium balances signify insufficiency of this nutrient.

The balance data reveal no extensive or certain effect of the administration of calcium supplements on the retention of this element.

In Period III the general record of Cows 3 and 4 was typical of what we have come to expect of fresh cows. They received sufficient amounts of feed to maintain nitrogen equilibrium, but suffered general loss of mineral nutrients.

Cows 7, 8, 9, 10, all either dry or nearly dry, and all late in the period of gestation, all stored nitrogen, sodium, and calcium, while positive balances also prevailed with the majority of the other elements, except for potassium and magnesium, with which 3 and 2, respectively, out of the 4 balances were negative.

*Sodium.*—Our arbitrary fixing of the sodium chloride intake at 0.007 of the amount of the grain, resulted in the provision of an allowance which was, perhaps, hardly sufficient, since 7 out of the 18 sodium balances, and 3 out of the 18 chlorine balances were negative; but, except for the case of Cow 3, Period II, which was manifestly underfed, the maximum loss of sodium was 2.5 gm. per day, and of chlorine 1.4 gm.; and the gains were much more numerous and extensive than the losses.

In consideration of the extreme mobility of sodium, and the size of the cow (not to mention the difficulty of estimating sodium with accuracy), we are not inclined to emphasize the significance of a maximum loss of 2.5 gm. of sodium per day; but to provide against the possibility of loss a larger intake might well be provided.

We incline to regard as more significant than any of the losses of sodium the positive balances of Cow 4, Period I, Cows 4, 7, 8, 9, and 10 in Period II, and Cows 7, 8, 9, and 10 in Period III, these positive balances, some large, some small, apparently resulting especially from a considerable intake, or low milk production, or advance in the period of gestation, or combinations of these influences which favor storage.

Comparing the sodium balances of Cows 3 and 4 in Period III, the loss of Cow 4, in spite of high intake, was in response to a general state of metabolism which caused losses of all elements studied, except nitrogen and arsenic. This condition was a high milk production, 61.36 pounds, on a ration which was insufficient in amount to support it.

The largest storage of sodium, 10.2 gm. per day, occurred with Cow 7, Period II, in which case we had a combination of high sodium intake, low milk production, and lateness ( $7\frac{1}{2}$  months) in the period of gestation.

*Chlorine.*—The three negative balances of chlorine appear to be due to the very high chlorine content of the first milk of a fresh cow (Cow 3, Period II), to very high milk yield (Cow 4, Period III), or to a very laxative diet (Cow 7, Period III). This last cow defecated, on an average, 12.4 times per day during the 16 days of this collection period. It was doubtful throughout this period whether she could be retained in the experiment.

*Potassium.*—13 out of 18 balances of potassium were negative. These losses were largely in the urine, the proportion of the total outgo which was in the urine being 67.3 per cent for cows in full milk, and 87.9 per cent for cows which were dry or nearly dry. These negative balances of potassium were never equal to more than a small part of the amount of the potassium in the urine. There was no suggestion, then, of deficient absorption of this element.

The 5 positive balances were all with cows which were dry or nearly dry, but not all of the dry or nearly dry cows stored potassium.

The sodium and potassium balances differed in 6 out of the 18 cases, there being no marked tendency for these to vary together. In these 6 cases the sodium balances were always positive and the potassium negative.

The high urinary outgo, in all cases, including those in which the balance was negative, indicates that these losses were not due to actual insufficiency of the intake. We are unable with certainty to interpret them. They may possibly be due to unexplained interelemental relationships in metabolism, or to as yet undefined periodic fluctuations.

*Calcium.*—In this fourth experiment on the same subject we have our first positive calcium balance. In this investigation 10 out of 18 balances are positive. In each of these 10 instances, the cows were dry, or nearly dry. All the cows which were dry or giving as little as 3.95 pounds of milk per day, and one giving 9.98 pounds, stored calcium. No others did so.

All cows in full milk, and one other, lost calcium. The maximum loss (Cow 5, Period I) was in a case of general underfeeding, the cow representing days 55 to 74 of lactation, and the balances all being negative with the exception of chlorine. The second and third largest losses were with Cows 4 and 3, Period III, representing days 24 to 33, and 30 to 39 of lactation, respectively.

In connection with our previous 42 balances, all negative, in spite of calcium intake varying from normal to excessive, all of the subjects being in full milk, this present evidence is of unmistakable significance and validity.

In dairy cows of good productive capacity parturition releases a flow of calcium in the milk which proceeds to such extent independently of the food supply of calcium that a loss of calcium from the body is normal, at least on rations composed of winter feeds, either dry or including corn silage, until such time that this impulse to secrete milk has largely spent itself. From sometime after the middle of the period of lactation until she goes dry, and while she is dry, she can store calcium from rations of the types specified.

We were unable to prevent loss of calcium, during liberal lactation, by the feeding of calcium supplements, and neither was there any certain advantage resulting from so doing in any case, that is, whether the cows were milking or were dry.

In our judgment calcium balances are of very uncertain significance as indicating the sufficiency of the calcium intake. The normal loss of calcium during lactation is undoubtedly due to the elimination of calcium in the milk, but the calcium loss is not closely in accord with the extent of the outgo by this channel. This loss appears rather to be the resultant of several factors, among which are the degree of the impulse to secrete milk, the power of the cow to digest, absorb, and assimilate calcium, and the ability of the cow to draw upon her mineral reserves.

The calcium and phosphorus balances agreed as to sign (+ or -) in 15 out of the 18 balances, in harmony with the obvious fact that the associated metabolism of this pair of elements is very much greater than their metabolism independent of each other.

It is worthy of note, and significant, that all of the 10 positive calcium balances are associated with positive nitrogen balances, and all of the 4 negative nitrogen balances are associated with negative calcium balances; that is, a state of metabolism involving nitrogen storage appears to be a favorable influence as affecting calcium retention, but in our earlier work we found that large nitrogen retention may occur coincident with calcium loss.

*Magnesium.*—15 out of the 18 balances of magnesium are negative. These losses occur mainly through the feces. Of the 3



positive magnesium balances all were associated with positive balances of both calcium and phosphorus, but there are 6 other instances of positive balance of calcium and phosphorus together, in which the magnesium balances are negative.

Thus, magnesium may be retained, when calcium and phosphorus are also retained, but in this experiment was lost from the body more commonly than it was retained, under these conditions.

In 7 out of 10 instances of positive balance of calcium there were negative balances of magnesium, but there were no cases of magnesium storage associated with calcium loss. These data raise the question as to whether those conditions which are favorable for calcium retention are to any degree unfavorable for magnesium storage.

*Phosphorus.*—Among the 18 phosphorus balances 11 were positive. As usual the metabolism of this element paralleled that of calcium; but also, as usual, there were exceptional instances which demonstrated their partial independence. 9 of the positive calcium balances were accompanied by phosphorus balances of the same sign.

1 positive phosphorus balance (Cow 1, Period I) accompanied extensive milk secretion, in harmony with previous observations that the conditions necessary for phosphorus storage are more readily attainable than those necessary for calcium retention, probably by virtue of the more extensive metabolism of phosphorus in tissues other than the skeleton.

While the intake of phosphorus was in no case less than 25 gm. there was normally less than half of a gram of phosphorus in the urine, though as much as 14.977 gm. of phosphorus were eliminated in the milk, associated with a positive phosphorus balance, and 25.328 gm. in a case in which the balance was negative.

In 3 instances (Cows 3, 4, 7, Period III) the usual amount of phosphorus in the urine was much exceeded. With Cows 3 and 4 this was apparently due to acidosis, caused by the high proportions of grain in the rations, resulting in relative deficiencies of mineral bases. In the case of Cow 7, the subsequent history suggests that the slight excess of phosphorus in the urine was due to functional derangement, the nature of which was not determined.

*Sulfur and Nitrogen.*—Of sulfur 7 balances were negative and 11 positive; and of nitrogen, 4 negative and 14 positive. 3 of

the 4 negative nitrogen balances were associated with negative sulfur balances under circumstances signifying, clearly, under-feeding (Cows 2 and 5, Period I, and Cow 3, Period II).

The sulfur and nitrogen balances were of the same sign in 13 out of 18 cases. The 3 cases above mentioned, in which both sulfur and nitrogen balances were negative, were characterized by general mineral insufficiency. The conditions unfavorable to sulfur and nitrogen storage doubtless added an unfavorable influence as affecting the retention of the other elements studied.

The 4 negative sulfur balances which were accompanied by positive nitrogen balances signify, at face value, that the nitrogen intake was more commonly sufficient for maintenance of equilibrium than the sulfur intake. This evidence of independence in nitrogen and sulfur metabolism may be due to the facts that some nitrogenous structures, hair, for instance, are much richer in sulfur than others, and that there was a more rapid growth of hair in one cow than in another.

That the general metabolism of sulfur is organic, and associated with nitrogen in protein compounds, is manifest, the sulfate excretion signifying for the most part, surely, the elimination of a protein catabolite. The negative balances of sulfur seem to signify an intake insufficient to the demands of body repair, growth of the fetus, and milk production.

*Arsenic.*—Our main interest in the metabolism of arsenic was to determine if it would be eliminated in significant amounts in the milk. The balances of arsenic answer this question in the negative. No arsenic was found in the milk, except a trace (0.00004 gm. per day) in the abnormal lacteal secretion of a cow which was virtually dry and was not being regularly milked. During the 16 day balance period this cow was milked only four times, as the condition of the udder required.

The amount of arsenic administered was a liberal dosage, 0.287 gm. of the element per day. So far as our evidence goes, then, the above amount of arsenic, fed to a cow, will not be eliminated in the milk in determinable amounts, but the fact that arsenic was retained in each case suggests that in a long continued use of arsenic there might be an accumulation of this element in the tissues such as would be eliminated in significant amount in the milk; but this is only hypothesis. Experiments covering this

point should be conducted. This apparent retention, however, may have been in part, at least, an undetermined elimination, as, for instance, by the skin.

Arsenic was eliminated in considerable amounts in both urine and feces, but, in all cases, in much larger amounts in the feces. The urinary outgo varied between 0.05566 and 0.0958 gm., the fecal outgo between 0.1022 and 0.1537 gm., and the retention between 0.047 and 0.124 gm. These amounts are of the element.

We see no indication at all that arsenic is effective as a calcium tonic, that is, that it is conducive to calcium retention. This does not mean, however, that it might not have such an action in pathological conditions or in the metabolism of a growing animal.

Further light on the metabolism of arsenic by cows is furnished by the work of Bloemendal,<sup>4</sup> and of Harkins and Swain.<sup>5</sup>

Bloemendal cited many conflicting statements concerning arsenic metabolism, and reported on experiments of his own. He found that cows in a normal, healthy condition did not eliminate arsenic in their milk, so long as it was given them in amounts below the point of toxicity. However, cows which had been given non-toxic doses previous to calving eliminated traces in the milk immediately after calving.

Harkins and Swain, showed in connection with a study of arsenic poisoning of livestock, as a result of eating forage contaminated by smelter fumes, that in cases of chronic poisoning by arsenic this element is unmistakably eliminated in the milk.

#### DISCUSSION OF RESULTS.

The most important addition to our knowledge of the mineral metabolism of the milch cow which is contributed by this experiment is that under the same conditions as to feeding, care, and experimental routine which have resulted, in this series of studies, in 49 negative balances of calcium during the liberal milk production of the first half of the period of lactation, without one exception, the subjects of this last experiment, which covered an entire year in the life of the cow, stored calcium as soon as the milk production had decreased to such an extent that the calcium outgo did

<sup>4</sup> Bloemendal, W. H., *Arch. Pharm.*, 1908, ccxvi, 599-616.

<sup>5</sup> Harkins, W. D., and Swain, R. E., *J. Am. Chem. Soc.*, 1908, xxx, 915-946.

not exceed their capacities to assimilate calcium, and also, invariably, when they had ceased to produce milk.

The exact point in the shrinkage of the milk flow at which it is possible for a cow to store calcium was not determined, but we are prepared to learn that this point is much above the production of 10 pounds of milk per day.

In the light of the results of our four studies on this subject, which cover, in a more or less continuous series of observations, the whole of the annual cycle of lactation and gestation, we are able to depict the calcium metabolism of a year in the life of a producing cow in the following terms.

Parturition turns loose a pent-up flood of nutriment which has been stored for the growth of the calf. This outpouring of mineral-rich food proceeds in large measure independently of the food supply; that is, if the food is sufficient to maintain the life of the cow she will produce milk even though this involves extensive drafts upon the tissues of the body.

This impulse to secrete milk has been greatly intensified by selective breeding. We have to do, therefore, not alone with nature's adjustments, but especially with the effects of man's interference with nature by the creation within the cow of an impulse to produce very much more milk than does the cow as nature made her.

Now, in intensifying this tendency, we have developed the power of the cow to draw upon the nutrient stores which compose her own tissues to such an extent that the results are often matters not only of practical importance but also of serious concern.

At some point between the middle and the end of the period of lactation, when the impulse to secrete milk has largely spent itself, the milk production comes to be more definitely related to and dependent upon the feed intake, and falls off, in amount, to such extent that retention of calcium, the dominant factor of the whole mineral system, comes to prevail.

In the meantime, under conditions of practice, the cow has been bred. The demand of the fetus for mineral substance is slight, in the light of the capacity of the cow to metabolize mineral substance in the elaboration of milk. True, toward the end of the period of gestation the fetus increases greatly its appropriation of mineral substance, but this factor remains a minor one in the mineral metabolism of the cow.

In the unimproved cow lactation ceases when the calf is weaned, long before the end of the next period of gestation, but with many cows of improved breeding the milk production may persist up to the time of parturition, and the history of cattle breeding embraces accounts of cows which have produced milk for several years after birth of a calf.

The more successful breeders have learned, however, that the feeding and management of the highly developed milch cow during the latter part of the period of gestation is a matter of critical importance, and it is in this connection that the results of this experiment are of greatest interest.

Our findings and the most successful practice both indicate that a cow must have a dry, resting period of sufficient length to permit the entire replacement of the preceding mineral overdraft, if the vitality, fecundity, and productiveness of the cow are to remain unimpaired; and to this end it is desirable that the dry cow be fed as liberally as practicable, without undue risk of milk-fever subsequent to the following parturition.

We have encountered, among practical dairyman, a belief that certain feeds stimulate milk secretion to an extent which they are inadequate to sustain. Alfalfa hay is said to have such a tendency. This may possibly be true in the sense that if the alfalfa is consumed liberally and is not accompanied by adequate amounts of carbohydrate nutriment its proteins may stimulate the cow to produce milk at the expense of a draft upon the body fat. Thus, the more liberal the feeding of alfalfa hay the heavier might be the milk production, with increasing emaciation of the cow.

A possible instance of such a situation is to be seen in the performance of Cows 3 and 4, Period III (see data below). Reference to Table VII, page 294, will show that these cows stored small amounts of nitrogen, but that the mineral balances were nearly all negative; and reference to Table III, page 287, shows that both cows were losing in weight. At the same time the milk production of Cow 3 increased continuously, while Cow 4 increased to her maximum and then began to recede. These balances covered days of lactation 30 to 39, and 24 to 33, respectively. It is conceivable that the nitrogenous components of the ration exerted an activating or stimulating influence as affecting milk yield.

*Daily Milk Yield of Cows 3 and 4 in Period III.*

Date.	Cow 3.	Cow 4.
	<i>gm.</i>	<i>gm.</i>
Mar. 23	23,755	26,960
" 24	24,529	27,627
" 25	24,312	27,745
" 26	24,073	28,525
" 27	24,330	27,890
" 28	24,944	27,894
" 29	24,975	28,820
" 30	25,284	27,873
" 31	25,279	27,554
Apr. 1	25,597	27,441

Meigs, Blatherwick, and Cary<sup>6</sup> after discussing our previous results in connection with like data announced the conclusion that

"The separate collection of urine and feces by attendants, as practiced in balance experiments on cows, produces a nervous disturbance in the animals which interferes markedly with the assimilation of calcium, and, to a less degree, with that of nitrogen and phosphorus."

We understand that the gains and losses of calcium and other mineral elements could not prevail indefinitely in the same relative amounts, one to another, as observed in some instances in this investigation; and also that the retention of calcium by cows under conditions of practice must be more extensive than as observed in our experiments, but we hesitate to ascribe these conditions to the cause assumed by Meigs and associates since we are without evidence as to the effect of any specific nervous influence, of the sort suggested, on calcium metabolism, and since there was no evidence of nervous disturbance in our experimental subjects.

Hart and associates<sup>7</sup> have shown that lactating goats made more efficient utilization of the calcium of the ration when the roughage was fresh, green oat plant than when it was dry oat straw, the balances of calcium, however, remaining negative.

<sup>6</sup> Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xl, 469.

<sup>7</sup> Hart, E. B., Steenbock, H., and Hoppert, C. A., *J. Biol. Chem.*, 1921, xlviii, 33; *Science*, 1920, lii, 318.

The senior author of this paper has reported observations<sup>8</sup> on a group of cows at the Ohio Agricultural Experiment Station, which have an important bearing on the subject of calcium metabolism.

Since the year 1911 this group of cows has been maintained on dry feeds alone, under the management of Mr. C. C. Hayden. Several of these individuals were born and raised to maturity without having had a bite of green feed. Quoting from our published observations on these dry-fed cows:

"This group of cows has grown to normal weights, and has produced and reared calves without marked or certain irregularity or abnormality. The milk production has been fair only, it being obvious that with normal treatment these cows would have given more milk. They do not have normally keen appetites, and some are easily forced off feed. They will not eat enough to support maximum milk production. They fall away during lactation a little more than is customary, but pick up again after going dry. These cows have been in noticeably less thrifty condition, as indicated by flesh and coats, than the balance of the herd, which goes to pasture, and it has been apparent that they crave something which they do not find in the ration.

"It appears, therefore, that the suggestion of Hart and associates is a matter of practical importance as relating to milk production, but that rations of dry feeds and silage, though probably deficient in some constituent, are not entirely lacking in any essential."

They ate steamed bone with avidity, which suggests that they may have been in a state of mineral depletion.

In the light of the work of Hart, with goats, and our observations on the group of dry-fed cows at the Ohio Station, it seems to us likely that shortage of pasture, and, therefore, of the "fresh-grass vitamine," rather than shortage of calcium and phosphorus, is the factor accountable for the unusual annual decrease in the yield of the cows of the main herd at the Beltsville Farm of the Department of Agriculture, as reported on by Meigs and Woodward.<sup>9</sup> These cows are said to have "little or no pasture." Their response to phosphate feeding, and to a prolonged dry period, seems phenomenal. It appears that these cows are comparable, in condition, to the dry-fed group at the Ohio Station, and that the conclusions of Meigs and Woodward as to the efficacy of feeding sodium phosphate, and the alternate feeding of grain and forage,

<sup>8</sup> Forbes, E. B., *Science*, 1920, lii, 467-468.

<sup>9</sup> Meigs, E. B., and Woodward, T. E., *U. S. Dept. Agric., Bull. 945*, 1921.

on separate days, apply rather to cows which are suffering from lack of pasture than to cows in general.

The difference between winter roughage and green forage, then, in the light of our present incomplete understanding, seems to be a secondary factor in the complex which causes fresh cows on winter rations to draw on their mineral reserves for calcium, the most important causes being the exaggerated impulse of the improved cow to secrete milk, and her limited ability to assimilate calcium. The ultimate cause of this limited ability to assimilate calcium has not been determined.

As indicating the possibilities of calcium storage, on dry winter rations without silage, we cite the case of Cow 4, Period II, page 292. This dry cow stored 14 gm. of calcium per day. The maximum amount of calcium eliminated in the milk, in this experiment, with this same cow, in Period III, after calving, was 29.4 gm., the cow producing 61.36 pounds of milk, with a negative balance of 12.3 gm. of calcium, the apparent assimilation of calcium from the ration being 17.1 gm.

The possibilities of calcium retention, and calcium utilization in the elaboration of milk, therefore, appear to be of similar magnitude. The storage in the first case exceeded the negative balance in the second. It would appear, then, that a dry cow can store calcium at a rate not unlike that at which she is apt to lose during heavy milk production on a good ration; in fact the brevity of the dry period suggests that under conditions of practice cows must store calcium much more rapidly, and earlier in the period of lactation, than we have as yet observed.

Two facts with reference to the utilization of calcium by the cow stand out with great prominence, first that the cow's ability to assimilate calcium from winter rations, either with or without the addition of organic or inorganic supplements, is more definitely limited (at least under experimental conditions), than is its ability to assimilate other nutrients; and second, that the freedom with which cows can draw upon the calcium of their own bones, and the extent of this draft in cases in which it is necessary, shows that, at least above a certain amount, the calcium of the skeleton is more readily available than is that of winter rations and calcium supplements fed in the usual way.



In regard to this whole matter of negative calcium balances in milking cows, this situation is perhaps a little more readily understandable if considered as related to the localized withdrawal of mineral salts from bone during pregnancy. The main facts as to this phenomenon are understood in connection with human physiology, in which the bone dissolution sometimes involves the teeth; and, in rare instances, the mineral disorganization proceeds even to the production of osteomalacia, which has been regarded, in this connection, as an uncontrolled accentuation of the normal osteoclastic changes of pregnancy.

In such an investigation as this which we discuss, one is puzzled by the appearance of negative balances which cannot possibly signify insufficiency in the supply of the nutrients in question. It is obvious that negative balances do not always have the same signification. In regard to these negative balances we suggest the following possibilities:

Negative balances may be due to deficiency in the supply of the nutrient; they may also be determined by negative balances of other elements or compounds, organic or inorganic (including vitamins), which are necessary to the utilization of the element in question; they may be periodic fluctuations, connected with the reproductive cycle, which would be revealed as such by the accounting for the nutrient in question during the whole of this period, the causes, therefore, lying entirely outside the ration; they may be fluctuations in extensive reserves, especially of the more highly mobile elements, in response to physiological antagonism, or "oppositeness" of function, in cases where excessive intake is to be disposed of, or where unusually extensive utilization involves abundant transfers; they may be due to inherited demands for the mobilization of greater amounts of a nutrient than the organism is able to digest or to assimilate, as in milk production, thus demanding drafts upon the reserves of the body.

The interpretation of a negative balance, then, may be a matter of some uncertainty, presenting opportunity for the exercise of judgment and the possibility of error.

*Practical Bearings.*—The practical significance of this subject of the mineral metabolism of the cow lies in the fact that milk production, on winter rations, involves considerable drafts upon the mineral substance of the skeleton.

As previously suggested (page 281), there are in connection with the behavior of cows a number of situations in which this mineral depletion may play a significant rôle.

Meigs and Woodward<sup>9</sup> have reported detailed observations on the behavior of cows, in this relation, which are of great interest and value. Whatever the extent of the part played by mineral depletion in the production of these states of functional derangement it is obvious that the maintenance of maximum productive activity requires that the mineral overdrafts occurring during the early part of the period of lactation must be repaid.

This restoration of mineral reserves occurs during the later part of the period of lactation, and especially during the dry period, and requires that the ration be at least moderately rich in mineral nutrients, especially calcium and phosphorus.

The advantage to be derived from the feeding of mineral supplements seems to us doubtful, but it is so easy to provide minerals in this form that the possibilities of benefit from so doing should be thoroughly investigated, under conditions of practice, not only during winter feeding but also during the season of pasturing.

The results of this investigation emphasize the necessity of a dry, resting period. The feeding during this time should be sufficiently liberal to permit the building up of extensive reserves of nutriment which shall protect the vitality of the cow and permit the full expression of her capacity to produce milk during the following period of lactation.

Provisionally we recommend, in harmony with the suggestions of Meigs and Woodward, liberal feeding during a dry period of 4 to 6 weeks for cows in a normal state of nutrition, and 8 weeks or more for cows in a state of depletion; also that during this period cows should be fed two to three times the amount of total nutriment and three to four times the amount of protein necessary for maintenance.

The primary opportunity for building up the nutrient reserves of the cow is during the growth of the heifer. Dairymen have debated the question as to the wisdom of liberal feeding during this period, with the preponderance of sentiment in the affirmative. The facts as to the mineral metabolism of the cow suggest the great desirability of making the most of the storage or constructive possibilities of the animal at this time.

A question raised by this investigation and Hart's observations on milch goats is as to the ability of a cow, in full milk flow, to maintain calcium equilibrium during the improved conditions for calcium utilization which prevail while she receives green feed. We anticipate an affirmative answer to this question, at least for cows of moderate productive capacity.

The results of this investigation apply, in a practical way, more to the dry-fed cow than to the cow at pasture, and more to matters of management and feeding together than to feeding alone.

As for the bearing of this investigation on the feeding of cows suffering from actual mineral shortage in the ration, the facts, as we have demonstrated them, emphasize the seriousness of such a shortage, but, unfortunately, we have little evidence as to the conditions under which such actual shortage prevails. By informal observation we understand that deficient supplies of minerals in the rations of cattle do prevail during certain more or less unusual conditions as to components of the ration and as to soil and season, but this knowledge is indefinite, and does not tell us what we need most to know, that is, whether rations of grain and gramineous roughage can supply the full requirement of the cow for minerals, or whether a certain proportion of leguminous roughage is necessary for best results. The final clarification of these points will require metabolism investigations with green forage of gramineous and also of leguminous origin.

Other practical bearings, suggested in our reports on the earlier experiments of this series, are that our chief concern in feeding practice is not so much to avoid definitely pathological conditions as to attain the maximum of efficiency, and that in the exigencies of practice, the slight losses of minerals observed while feeding the high-class rations of our experimental work may be magnified, by unfavorable ration conditions, into considerations of importance.

It is further suggested that the skeletal development is dependent on the forage, since grains are very deficient in calcium; that the mineral content of the forage varies widely in accord with the natural composition or artificial fertilization of the soil; that there are seasonal effects on the mineral nutrients of the forage; that food shortage, deficiency of mineral nutrients in the forage, and other hardships, may react unfavorably on the mineral nutrient reserves, in this way becoming contributory causes of

sterility, abortion, and malnutrition of the bones; and, finally the specific bearings of the whole subject on general farm practice, especially as favoring the production of legumes, are pointed out.

In our experimental work the calcium balances have been determined by the functional activity of the cow rather than by the nature of the calcium compounds offered in the ration. No basis was developed for the preference of one calcium supplement to another. On presumptive evidence alone, then, we suggest, for practical experimental purposes, the feeding of calcium phosphate in the form of steamed bone.

Steamed bone intended for feeding purposes should be promptly dried, and specially handled in a way to make it safe and acceptable; and, to facilitate its solution in the digestive secretions, it should be finely ground, preferably through a 20 mesh screen. Cows will eat fertilizer bone, but, in consideration of the methods by which it is ordinarily produced and handled, we do not recommend it as a feedingstuff.

Cattle will take steamed bone readily, either mixed with the feed, or offered at free will mixed with one-fourth as much common salt. From 2 to 6 ounces per day appear to be reasonable amounts to feed.

We have not tested the ideas of Meigs and associates that sodium phosphate is a more effective mineral supplement than calcium phosphate, and that the alternate feeding of grain and roughage on separate days (the phosphate being fed with the grain) is especially conducive to calcium and phosphorus retention, but we have shown that, under the conditions of a metabolism investigation, a dry cow stored as much as 14 gm. of calcium per day without the provision of sodium phosphate or the practice of the system of alternate feeding.

#### SUMMARY.

The calcium metabolism of the milch cow, while fed on winter feeds, is characterized by rapid loss from the body during the early part of the period of lactation, changing to retention late in the period of lactation, by continued retention during the dry period, with most rapid storage at the end of the period of gestation.

The principal factors determining the loss of calcium during the early part of the period of lactation are the impulse to secrete

milk, as accentuated by selective breeding, and a limited ability to assimilate calcium.

In the course of this investigation there were 49 negative calcium balances during liberal milk production, without one exception. The largest milk production with which there was calcium retention was 9.98 pounds. It is considered likely that calcium retention can occur during more extensive milk production.

The dry cow, on dry feeds, can store calcium at a rate at least equal to that at which the fresh cow, on dry feeds, loses calcium.

The loss of calcium from the body appears to be a prominent factor in the nutritive depletion and the functional derangement of the overtaxed milch cow.

The ability of a cow to assimilate calcium is much more definitely limited than her ability to assimilate nitrogen.

The calcium of the bones is more readily available, for purposes of milk elaboration, than the calcium of the ration and of mineral supplements.

It is not clear that supplemental calcium is utilized, either during lactation or during the dry period.

There was no evidence of a tonic effect of Fowler's solution in relation to calcium or other mineral retention.

A marked but not complete interdependence of calcium and phosphorus in metabolism was manifest. Phosphorus may be stored during liberal milk production; calcium seems never to be stored under these conditions, at least on winter rations.

A positive nitrogen balance probably favors calcium retention to a slight extent, but the measure of independence existing in the metabolism of nitrogen and the mineral elements is prominent.

There is a considerable but not complete interdependence of nitrogen and sulfur in metabolism.

The average apparent digestibility of the protein of the experimental rations was 66.23 per cent; of the nitrogen-free extract, 78.64 per cent; of the ether extract, 65.02 per cent; and of the crude fiber, 36.33 per cent.

The proportionate elimination of minerals by urine and feces bears no definite relation to mineral balances. Many negative mineral balances were determined which could not signify insufficient intake.

Five possible interpretations of the significance of negative balances of mineral elements are suggested (page 310).

The composition of milk including the colostrum suggests that its laxative character is due to physiological "salt action."

The milk of the cow was found not to contain arsenic, during the administration of 0.287 gm. of the element per day, in the form of Fowler's solution.

The milk toward the end of the period of lactation was found to differ from that of the middle of this period by being richer in nitrogen and in all of the minerals except potassium, which was distinctly low. Sodium and chlorine were in greatest excess.

Calcium was eliminated in the urine in the smallest amount by the freshest cows.

Chlorine excretion in the urine was low in the milking cows and high in the dry cows.

A high proportion of grain in the ration produced a marked increase in the urinary outgo of phosphorus, apparently as an expression of acidosis.

The physiological antagonism of sodium and potassium, and of calcium and magnesium, is not discernible in the metabolism of practical rations and the considerable variety of mineral supplements used in this investigation (including the three previous studies).

The results of this investigation suggest the desirability of building up extensive mineral reserves in growing heifers by liberal allowance of feeds rich in mineral nutrients, and also the importance of a dry, resting period of adequate length to permit the restoration of all previous nutrient overdrafts, with liberal feeding during this period.



## STUDIES ON URIC ACID.

### I. EXAMINATION OF THE VARIABLES IN THE FOLIN AND WU URIC ACID METHOD.

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Uric acid, although one of the earliest isolated products of metabolism, has offered numerous difficulties for quantitative estimation. Of the older methods, that of Salkowski (1) gave the most reliable results. But, until Folin and Denis (2) in 1913, by painstaking research, developed a colorimetric determination, no method was available for estimating, even with relative accuracy, such small concentrations of uric acid as occurred in the blood. In their original method acetic acid was used to coagulate the proteins and the uric acid precipitated by means of silver lactate magnesia mixture. The silver urate so precipitated was decomposed with hydrogen sulfide water and the uric acid read colorimetrically from the blue color produced by the reduction in alkaline solution of a specially prepared phosphotungstic acid reagent. The recovery of uric acid by this procedure, according to Folin's data, averaged about 94 per cent. In 1914 Steinitz (3) carried out a detailed study of the Folin method, but made only very minor alterations in the procedure. His recoveries were not nearly as good as those of Folin, only averaging 80 per cent. The next improvement was that of Benedict (4) in 1915, who employed a double protein precipitation, using first acetic acid and colloidal iron. This procedure furnished a protein-free filtrate which matched the color of the standard very closely. Benedict and Hitchcock (5) also eliminated the use of hydrogen sulfide water, dissolving the silver urate precipitate with potassium cyanide. As to the actual amount of uric acid recovered no data are presented, merely the



statement that "results obtained for the most part closely parallel those obtained by the Folin-Denis method." Landmann (6) using Benedict's modified procedure reports about an 85 per cent recovery, while Chace and Myers (7) report a recovery of about 88 per cent.

These methods, although more rapid and accurate than any hitherto in existence, are rather tedious and required for maximum efficiency at least 20 cc. of blood. There were no marked changes in the procedure until 1919 when Folin and Wu's (8) "System of blood analysis" was published. In that paper is included a rapid method of uric acid determination on a very small quantity of blood (4 to 5 cc.). The new features are the novel protein precipitant, tungstic acid, and the decomposition of the silver urate precipitate by means of dilute hydrochloric acid solution. Also the long tedious evaporation of the earlier method is avoided. This new procedure is used now in a large number of clinical laboratories throughout the country, but as far as the writer is aware, a detailed test as to its limits of accuracy have not been reported by other investigators. Furthermore, the original paper, being compressed by the wealth of material covered, obscures details of technique the importance of which can only be realized and appreciated by a careful study of the process.

Finally, it must be remembered that it is not justifiable to apply an analytical procedure to all sorts of varying conditions as blood analysis represents, without knowing accurately under what conditions the method is quantitative and what variables influence its accuracy. After all, analytical methods are only carefully controlled chemical processes which must regularly produce yields as near to 100 per cent as possible, and we must know within a few per cent how consistent and what is the average variation of that yield. With this general view-point in mind the following experimental data were collected.

#### EXPERIMENTAL PART.

*Method of Analysis Employed.*—The directions outlined by Folin and Wu (8), of which a brief digest is given below, were followed as closely as possible.

5 cc. of whole blood are hemolyzed with 35 cc. of water and the proteins precipitated with 10 cc. of the tungstic acid reagents (5 cc. of 10 per cent sodium tungstate and 5 cc. of  $\frac{2}{3}$  N sulfuric acid). To 20 cc. of the filtrate are then added 4 cc. of a solution of 5 per cent silver lactate in 5 per cent lactic acid and the suspension is centrifuged. The clear supernatant liquid is poured off and the precipitated silver urate admixed with other silver salts decomposed with 10 per cent sodium chloride in 0.1 N hydrochloric acid. The mixture is again centrifuged and the clear fluid poured into a 25 cc. volumetric flask, any suspended silver chloride being dissolved by a little sodium cyanide. An excess of sodium carbonate (20 per cent) and the uric acid reagent are then added, the solution is made up to volume with distilled water and read against the color developed by 1 cc. of the standard uric acid (0.1 mg. per cc.) diluted to 50 cc.

*Reagents.*—The various reagents were prepared according to Folin and Wu's (8) directions. In all cases c. p. grades of chemicals were chosen and all solutions analyzed before using. Percentage by weight was employed and the solutions were within less than 3 per cent of the concentrations designated by Folin.

*Uric Acid Standard.*—The uric acid was dissolved in a 0.4 per cent lithium carbonate solution and preserved with sodium sulfite. Considerable difficulty has been encountered, contrary to Folin's statement that in "unopened bottles we expect the uric acid to keep for many years." While at times the standard solution has been stable over periods of 3 months, several bottles have decomposed in less than 24 hours. This sudden decomposition is probably due to bacterial infection since almost quantitative (90 to 95 per cent) conversion into urea had taken place. The same sample of uric acid was used throughout this work so as to obtain accurate relative data. Investigations are in progress on the effect of the purity of uric acid on its stability in dilute solution as well as to the extent with which the other commonly occurring purines and pyrimidines are evaluated in the uric acid method.

*Apparatus.*—A Duboscq colorimeter was used. All readings were made in a dark room with artificial illumination. The operator remained in the dark at least 2 to 3 minutes before readings were taken and the colorimeter was tested at the begin-

ning and end of each series for equal brightness of field. The color to be read is a very pure blue, and, as regards the human eye, at a very unsensitive part of the spectrum. Hence, although with practice, accurate readings can be obtained in daylight, nevertheless, the dark room affords much more consistent results. Each reading must be made rapidly since the eye becomes fatigued in a few seconds. Occasionally a yellow glass over the eye-piece has been found to be very restful, identical readings being obtained, remembering that it is intensity and not shades of the fields that is being compared. The final accepted reading was the average of six to ten successive ones. A greater accuracy than 3 per cent could not be expected from the instrument employed.

All pipettes used were calibrated for delivery and the same series of freshly calibrated volumetric flasks used throughout these experiments. This assured constant relative data.

#### *Variable Studies.*

*Relation between Depth of Color and the Uric Acid Present.*—The primary assumption in any colorimetric method of analysis employing an instrument with adjustable cells is that the variation in scale reading (*i. e.* amount of light absorbed by different depths of the colored solution) is inversely proportional to the amount of color-producing substance present in solution. This assumption is not always true and frequently correction curves have to be plotted. In fact, Steinitz (3), in studying the Folin and Denis (2) uric acid procedure, applied correction curves to their values, but both the early and later publications of Folin and his coworkers assume that this is not necessary, although no direct experimental figures are presented. To evaluate the magnitude of this possible error the data tabulated below were collected.

60 to 100 mg. of uric acid were weighed in a small beaker, dissolved in 10 to 15 cc. of 0.4 per cent lithium carbonate solution, and made up to 1 liter. 2 cc. of this solution were read directly without the precipitation of the silver salt. Table I summarizes the results obtained.

These data clearly demonstrate that, within the limits of the colorimeter (2 to 3 per cent), the concentration of uric acid can be calculated directly from the observed colorimetric readings.

*Conditions for the Quantitative Precipitation of Uric Acid.*— Since in most instances it is impracticable to determine uric acid by direct reading of a solution due to interfering compounds, some method of separation must be chosen. The precipitation of silver urate has been adopted as the simplest and most efficacious method, but nowhere are clearly outlined the limiting conditions under which this salt is precipitated quantitatively. All the early investigators precipitated the uric acid in an ammoniacal solution in the presence of ammonium chloride or magnesium sulfate. Each investigator varying the concentration of ammonia

TABLE I.  
*Relation between Colorimeter Reading and Uric Acid Present.*

Date.	Uric acid added per 100 cc.	Uric acid found per 100 cc.
<i>1921</i>	<i>mg.</i>	<i>mg.</i>
Aug. 25	6	5.9
" 26	10	9.0
" 26	10	10.4
" 26	10	11.0
" 26	10	10.0
Sept. 23	7	6.8
" 29	7	6.7
" 29	7	7.0
" 30	7	6.9
Oct. 1	7	6.9
<i>1922</i>		
Jan. 2	2	2.0
" 4	2	1.9
" 6	3	2.8

and magnesia mixture. That these were far from quantitative conditions is shown by the statement of Folin that by the Sal-kowski method of precipitation (magnesia mixture and silver lactate) a 50 per cent loss is encountered, when 0.1 mg. of uric acid is precipitated from 10 cc. of solution. Folin and Wu employed a non-ammoniacal solution for the precipitation of uric acid, their blood filtrates being almost neutral.

Experiments showed that a solution for the quantitative precipitation of uric acid must be neutral (litmus or brilliant yellow papers as indicators) before the addition of the silver lactate

reagent. In fact it is safer to have the solution slightly alkaline with ammonia (just red to brilliant yellow paper) than faintly acid. A greater excess than 0.2 cc. of concentrated ammonia in 25 cc. of filtrate should be avoided and under no condition should the solution be alkaline after the addition of the silver lactate reagent. The great sensitivity of the silver urate to mineral acids is shown by the fact that from 25 cc. of a  $N/57$  sulfuric acid solution only 16 to 20 per cent of the added uric acid could be recovered. Fortunately, however, the solubility of silver urate is not appreciably affected by relatively high concentrations of lactic acid, and the addition of 4 to 5 cc. of a 5 per cent solution has no effect.

TABLE II.

*Recovery of Uric Acid in Neutral Solutions.*

Date.	Uric acid present per 100 cc.	Uric acid found per 100 cc.	Recovery.
<i>1921</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
Sept. 30	6.8	6.6	97
" 30	6.8	6.6	97
" 30	6.8	7.0	103
Oct. 1	6.8	6.7	98
" 1	6.8	6.6	97
" 1	6.8	6.7	98
" 1	6.8	6.7	98
<i>1922</i>			
Feb. 4	2.0	2.0	100
" 5	2.0	1.9	95

To prove that a quantitative recovery of uric acid under the above mentioned conditions was consistently possible, one sample of a pure uric acid solution was read directly while another 2 cc. portion of the same solution after the addition of 4 cc. of the tungstic acid reagent (to parallel the conditions of the blood filtrate) was neutralized with ammonia and precipitated with 3 cc. of silver lactate reagent. The efficiency of the process is clearly outlined in Table II.

It will be observed that with large as well as small amounts of uric acid there is a very satisfactory recovery of uric acid.

*Effect of Temperature on the Precipitation of Uric Acid in Neutral Solutions.*

The precipitation is complete at ordinary room temperatures and no special precautions need be taken to cool the solution below 26°C.

*Time Necessary for Complete Precipitation.*—If the silver lactate is added slowly with stirring, the coagulation and precipitation are complete in from 7 to 10 minutes. Precipitation is complete when the precipitate has settled spontaneously and the supernatant liquid possesses only a faint opalescence. From 2 to 3 minutes centrifuging gives a water-white filtrate.

*Volume in Which Precipitation Is Made.*—The volume of the solution precipitated was 20 to 28 cc. and all data apply only within these limits.

TABLE III.  
*Effect of Temperature.*

Date.	Room temperature 26°C.	0-5°C.	Recovery.
1921	mg. per 100 cc.	mg. per 100 cc.	per cent
Sept. 30	6.6	6.6	97

*Effect of Coagulation of Blood Proteins by Tungstic Acid on the Recovery of Uric Acid.*—This phase of the problem can now be considered since the previous experiments have demonstrated that uric acid can be recovered quantitatively from a solution, if properly precipitated. Any loss that occurs must therefore involve the coagulated protein. In the earlier method the absorption of uric acid by the protein precipitate was practically negligible, due to the hot filtration, thorough washing, and the large amount of sample used. However, in the new procedure no washing of the protein precipitate occurs and, due to the small sample of blood (4 to 5 cc.), even a small retention by the precipitate would introduce a tremendous error. In fact, upon this factor now hinges the final success of the analytical procedure as applied to blood. Folin (8) only states that large traces of uric acid are not carried down with the blood proteins during the coagulation process, but again no figures are available as to the amount of acid recovered.

To settle this point sheep blood was chosen since it contains a negligible quantity (9) of uric acid (about 0.05 mg. per 100 cc.). To 5 cc. of sheep blood was added a measured amount of uric acid solution, the value of which was determined by direct reading in the colorimeter. The solution of sheep blood and uric acid was treated exactly according to Folin's directions, as outlined in the first part of the experimental part of this paper. For each series of determinations a 5 cc. sample of sheep blood was also analyzed to see that no uric acid was obtained from that

TABLE IV.  
*Recovery of Uric Acid by Folin's Procedure.*

Date.	Uric acid present per 100 cc. of blood.	Uric acid found per 100 cc. of blood.	Recovery.
<i>1921</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
Oct. 7	11	8.6	78
" 7	11	9.0	80
" 12	10	7.3	73
" 18	6.6	4.7	71
" 18	6.6	4.9	74
" 18	6.6	5.0	76
" 18	6.6	5.1	78
<i>1923</i>			
Feb. 4	2.0	1.6	80
" 4	2.0	1.7	85
" 5	2.0	1.5	75
" 5	2.0	1.5	75
" 5	2.0	1.4	70
" 6	2.0	1.6	80
" 6	2.7	2.1	76
Average .....			77

source. Also from time to time the original uric acid solution was precipitated in the absence of the blood. The values so obtained checked the direct reading within 2 to 3 per cent. The results are tabulated in Table IV.

These data indicate that in Folin and Wu's procedure a 20 to 25 per cent loss of uric acid occurs. This loss is practically constant if experimental conditions are duplicated each time, and must be due to absorption of mechanical retention by the coagulation and protein precipitation. These data also confirm

Folin's statement that a relatively large loss of uric acid does not occur and from the present clinical significance of the determination might be neglected, but from the standpoint of a quantitative method of analysis it is not justifiable if there is any means of increasing the yield. A simple modification of the uric acid procedure for this purpose will soon be communicated in the following paper.

In connection with Table IV criticisms have been made that this loss of uric acid may be due to three causes: (a) Addition of large amounts of uric acid (three times normal in the first part of the table), and that with a normal amount of uric acid quantitative results can be obtained. (b) That the sodium tungstate and sulfuric acid were not properly adjusted. (c) That the blood contained too much sodium oxalate.

On the basis of available literature Folin and Wu do not in any way emphasize the idea that their method is only for uric acid within the normal range (1 to 3 mg.). In fact the contrary is to be inferred from the following statement:<sup>1</sup> "As a working principle or guide in this search we have first of all required that the procedure employed must permit the quantitative recovery of at least 10 mg. of uric acid and creatinine when added to 100 cc. of sheep, beef, or chicken blood . . ." And again:<sup>2</sup> "As much as 20 mg. of uric acid may be added to 100 cc. of blood without incurring any loss by absorption." However, to ascertain definitely if a small amount of uric acid could be recovered more satisfactorily the latter half of the data in Table IV was collected. In no case could a quantitative recovery be observed although the average recovery is slightly better than with the larger quantities of uric acid. The final conclusion still stands that in the method outlined by Folin and Wu there is an appreciable loss of uric acid decreasing slightly when small amounts of uric acid are present.

The necessity of the careful adjustment of sodium tungstate and sulfuric acid was realized. If these solutions are not equivalent within very narrow limits extremely erratic results may be obtained. The results of the study on the adjustment of the protein precipitant will be reported in a future communication.

<sup>1</sup> Folin and Wu (8), p. 82.

<sup>2</sup> Folin and Wu (8), p. 83.



The data presented in Table IV were obtained from blood filtrates having a normality of N/300 to N/500 (Folin's blood filtrates are reported as about N/500).

The effect of sodium oxalate was eliminated in these experiments since fresh defibrinated sheep blood was used. No oxalate at all was present. In human blood, a neutral, dehydrated sodium oxalate was employed. The amount was weighed on an analytical balance so as to avoid an excess.

*Recovery of Uric Acid Using Trichloroacetic Acid.*—In reviewing the literature on uric acid it seems strange that no investigations were recorded employing trichloroacetic acid as the protein precipitant. The investigation of this would be of pertinent interest since this reagent is used so frequently, and, till tungstic acid was introduced, was the standard for protein nitrogen determinations.

TABLE V.  
*Recovery of Uric Acid Using Trichloroacetic Acid.*

Date.	Uric acid present per 100 cc.	Uric acid found per 100 cc.	Recovery.
1921	mg.	mg.	per cent
Nov. 1	5.9	2.8	48
" 1	5.9	3.1	52
" 4	6.7	4.4	66

5 cc. of sheep blood containing known amounts of uric acid were hemolyzed with 15 cc. of water and 20 cc. of a 5 per cent solution of trichloroacetic acid added and then 10 cc. more distilled water, giving, as in Folin's method, a total volume of 50 cc. After shaking and standing for 5 to 10 minutes the mixture was filtered and 20 cc. of the clear filtrate were neutralized with ammonia and the uric acid was determined in the usual manner, with the results shown in Table V.

It will be observed that the results seem to be rather erratic and only about a 50 per cent recovery of uric acid is obtained as against 75 per cent by the tungstic acid precipitation method. This experiment is an added confirmation of the remarkable properties of Folin's protein precipitant. These data are also of interest when viewed in the light of the total non-protein nitrogen recovery, by the trichloroacetic acid method, particularly

so when one notes that even with a 25 per cent increased yield of uric acid, Folin's non-protein nitrogens by the tungstic acid method are consistently 10 per cent lower than by the standard trichloroacetic acid precipitation. Of course, ordinarily the uric acid nitrogen is such a small fraction of the total that its effect is almost negligible, but in many pathological cases this is not the case.

#### SUMMARY.

1. A review of the literature shows that Folin and Wu's recently announced method for the determination of uric acid is the most rapid and accurate available for small quantities of blood.

2. The average uric acid recovery recorded in the literature is only 80 to 85 per cent.

3. Experimental data have been presented showing that: (a) Within the accuracy of the colorimeter (2 to 3 per cent) the readings are proportional to the amount of uric acid present without applying any corrections; (b) uric acid must be precipitated in neutral solution by silver lactate and that only under these conditions are consistent quantitative results obtained; (c) the precipitation of uric acid is very sensitive to mineral acid, but practically unaffected by lactic acid; (d) temperatures below 26°C. have no effect on the solubility of silver urate; (e) recovery by Folin and Wu's technique is consistently about 75 per cent; (f) the loss must be due to mechanical retention or absorption by the precipitated proteins; (g) by using trichloroacetic acid as the protein precipitant only a 50 per cent recovery of uric acid could be obtained. The relation of this fact to the non-protein nitrogen values was pointed out.

4. The uric acid precipitation by silver lactate can be extended to other solutions, provided precipitation conditions outlined are adhered to.

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## STUDIES ON URIC ACID.

### II. A MODIFICATION OF THE FOLIN AND WU URIC ACID METHOD.

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In the previous paper (1) it was shown that by the technique of Folin and Wu only a 75 per cent recovery of uric acid was obtained and that this loss occurred in the coagulation and precipitation of the blood proteins. To avoid this loss several experiments were tried, but none was satisfactory until heating of the solution before filtration was attempted. Folin and Wu (2) in describing their new protein precipitant mentions that, while heat may be resorted to, it is not recommended until some compelling advantage is gained. The advantage has arrived, namely increase in yield of uric acid to 93 per cent as well as rapidity of operation, due to increased speed of filtration.

During the course of this investigation it has also been found practicable to combine three of Folin's reagents into one solution; thus, greatly simplifying manipulative details. The solutions combined are the sodium sulfite, sodium cyanide, and sodium carbonate, and the concentration of the single solution has been so adjusted that the volume used (5 to 10 cc.) contains the same proportions of each reagent as recommended by Folin. This single solution keeps well, as one batch has been in use 3 months and still gives satisfactory results.

To the use of a hot coagulation of the blood proteins for the determination of uric acid at least two main objections might arise. First the heating process may liberate loosely combined uric acid or other color-producing substances which would account for the difference in results. This is not the case since sheep blood gave no increased color when analyzed by the heating

procedure and, furthermore, at no time could there be recovered from the blood a larger amount of uric acid than had been added to it. That this same conclusion applies to human blood is apparent from Table I.

It will be noted that in no case is the gain in yield by the heating process greater than is commensurate with the increased efficiency of the new method; *i.e.*, 18 to 20 per cent.

TABLE I.

*Comparison of Cold and Hot Coagulation of Proteins in Human Blood.*

Date.	Cold (Folin and Wu's method).	Hot coagulation per 100 cc.	Difference.
<i>1921</i>		<i>mg.</i>	<i>per cent</i>
Oct. 21	1.9	2.4	20
" 22	7.2	9.2	22
Nov. 2	0.75	0.90	17
" 3	3.3	4.1	19
" 3	1.9	2.4	20
" 8	2.8	3.4	18
Average increase.....			19

TABLE II.

*Comparison of Volumes of Mixture Before and After Heating.*

Volume before heating.	Volume after heating mixture for 5 to 7 minutes.
<i>cc.</i>	<i>cc.</i>
50	48.5
50	49.5
50	49.5
50	50.0

Secondly, it might be argued that the gain in yield is due to the concentration of the solution by evaporation during the heating process. Experiments indicate that with the type of flask used (which acted as a reflux condenser) and the short time period (5 to 10 minutes) loss of volume by evaporation was negligible. These data are presented in Table II.

Next, the efficiency and reliability of the new method were studied by adding known amounts of uric acid to separate samples of sheep blood and then analyzing the filtrates. The experimental data are tabulated in Table III.

These results clearly indicate that the method is consistent within the limits of accuracy of the colorimeter and that an average recovery of 93 per cent of the uric acid present can be obtained in contrast to 75 per cent by the cold precipitation of Folin and Wu. Not only that, but the filtration of the hot solution takes, with a properly adjusted paper at the longest 7 minutes and generally less than 5 while for the cold filtration 45 to 60 minutes are necessary. An added advantage of the hot filtration is that 3 cc. of blood may be used for analysis where necessity demands.

TABLE III.  
*Recovery of Uric Acid by the New Procedure.*

Date.	Uric acid added per 100 cc.	Uric acid found per 100 cc.	Recovery.
<i>1921</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
Oct. 20	6.7	6.1	92
" 24	4.7	4.3	92.5
" 24	4.7	4.2	90.3
" 24	4.7	4.4	94.5
Nov. 6	7.8	7.3	93.6
" 6	7.8	7.5	96.0
" 6	7.8	7.3	93.6
" 6	7.8	7.1	91.0
<i>1922</i>			
Feb. 8	2.7	2.5	92.6
" 5	2.0	1.9	95.0
" 6	2.0	1.8	90.0
Average.....			93

Having established that the hot coagulation and filtration of the blood proteins are practicable in the determination of uric acid the procedure adopted in this laboratory will be fully outlined. Many of the details, of course, are identical with those described by Folin and Wu, and for the sake of clearness will bear repetition.

#### *Reagents.*

1. *Uric Acid Standard.*—This was prepared exactly according to the directions of Folin with the addition that a few cc. of amyl alcohol for preservative purposes were added.

2. *Protein Precipitant*.—Prepared as described by Folin and Wu consisting of a 10 per cent solution of sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ) and a solution of  $\frac{2}{3}$  N sulfuric acid. This must be so adjusted that the blood filtrate is N/300 to N/500.

3. *Dilute Ammonia*.—14 cc. of concentrated ammonia diluted to 500 cc.

4. *Silver Lactate Reagent*.—5 per cent silver lactate in 5 per cent lactic acid.

5. *Silver Salt Decomposition*.—A 10 per cent solution of sodium chloride in 0.1 N hydrochloric acid.

6. *Combined Reagent*.—The following amounts of c.p. chemicals are dissolved in warm water, made up to 1 liter, and filtered:

	gm.
Sodium sulfite (anhydrous).....	20.0
“ carbonate “ .....	120.0
“ cyanide (100 per cent NaCN basis).....	5.0

7. *Uric Acid Reagent*.—The reagent recommended by Folin and Denis was used; *i.e.*, the heating of 100 gm. of sodium tungstate, 80 cc. of phosphoric acid, and 70 cc. of water for 2 hours and then diluting to 1 liter.

### *Method of Analysis.*

5 cc. of oxalated (excess oxalate *must* be avoided) whole blood are measured with a Folin pipette into a 125 cc. long, narrow necked Florence flask. The blood is laked with 35 cc. of water and then 5 cc. of sodium tungstate, followed by 5 cc. of the  $\frac{2}{3}$  N sulfuric acid are added. The flask is *vigorously* shaken and after standing 5 to 10 minutes (when the color *must* be a chocolate brown), immersed in a boiling water bath (from 95 to 98°C.) for 5 to 8 minutes. After this period it is removed, shaken gently, and filtered immediately. To 20 cc. of the filtrate (cooled to room temperature) in a 50 cc. centrifuge tube are added 0.5 cc. of the dilute ammonia and then with stirring 3 cc. of the silver lactate reagent. After standing 10 to 15 minutes the solution is centrifuged for 2 minutes and the *clear* supernatant liquid carefully poured off. The residue is then thoroughly triturated with 2 cc. of the 10 per cent sodium chloride in 0.1 N hydrochloric acid, the stirring rod and sides of the tube washed with 10 cc. of

water and again centrifuged. The clear liquid is now poured into a 25 cc. volumetric flask.

Next pipette 1 cc. of the standard uric acid into a 50 cc. volumetric flask, washing down the sides with 4 cc. of the 0.1 N hydrochloric acid and 20 cc. of water. Then add with shaking 10 cc. of the combined reagent to the standard, and 5 cc. to the unknown solution. Allow to stand for at *least* 10 minutes, and to the standard (50 cc. volumetric flask) add 1 cc. and to the unknown (25 cc. volumetric flask) 0.5 cc. of the uric acid reagent. Shake, let stand 3 to 5 minutes, dilute to the proper marks, and read in the colorimeter. As previously pointed out readings are best made in a dark room and the average of six to ten readings is taken as the correct value.

#### *Calculations.*

$$\text{Setting of standard (either 10 or 20 mm.)} \times \frac{\begin{array}{c} \text{mg. of uric acid in stand-} \\ \text{ard solution used for} \\ \text{colorimetric comparison} \\ \text{(usually 0.1 mg.)} \end{array} \times 50}{2 \text{ (reading of unknown)}} = \frac{\text{mg. of uric acid per}}{100 \text{ cc. of whole blood.}}$$

#### *Precautions.*

1. It is important that the sodium tungstate and  $\frac{2}{3}$  N sulfuric acid be properly adjusted so that an almost neutral blood filtrate be obtained (0.005 to 0.002 N). The solution must be analyzed before using. This is most easily accomplished by titrating one against the other, using methyl orange as indicator. 5 cc. of the sodium tungstate should be equivalent to about 3.0 to 3.3 cc. of the  $\frac{2}{3}$  N sulfuric acid.

2. In several instances for reasons yet unknown (particularly in cases of eclampsia with high uric acid) the hot blood filtrates possess a turbidity or opalescence. This, however, does not interfere with the uric acid determination and the final solution is water-white.

3. As first pointed out by Benedict and Hitchcock (3) (for the hydrogen sulfide water decomposition) thorough breaking up of the precipitated uric acid is absolutely essential. This point is just as vital in the hydrochloric acid decomposition of the present method and great care must be exercised that all particles are loosened from the bottom of the centrifuge tube and brought into intimate contact with the acid.



4. The dilution of the standard uric acid to about 20 cc. before the addition of the combined reagent is essential since it prevents the formation of the fine white precipitate which often takes place when the undiluted solution is allowed to stand.

5. Do not fail to add the 4 cc. of 0.1 N hydrochloric acid to the standard otherwise the full color may not be developed.

#### SUMMARY.

1. A method has been presented by which the yield of uric acid from the blood has been increased to 93 per cent.

2. This new modification is simple and shortens the time cycle as well as manipulative details of the original procedure.

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## A SAPONIN FROM AGAVE LECHUGUILLA TORREY.

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*Agave lechuguilla* Torrey, known among the Mexicans as "lechuguilla" or "amole de lechuguilla," grows abundantly on the limestone highlands of western Texas and along the Rio Grande as far east as Presidio, extending into New Mexico and Mexico, where, according to Michotte (1), it grows wild in abundance in the states of Tamaulipas, Chihuahua, Cahahulla, Hidalgo, and Nuevo Leon. It is of interest to note that different forms of agave have been called "*lechuguilla*." Watson (2) refers to "*lechiguilla*" or "*lechuguilla*" as the native name of *Agave guttata* and *Agave variegata*. According to Kew Bulletin (3), these species belong to quite another group, different from *Agave lechuguilla*. The statement is made that

"We may look upon *Agave lechuguilla* Torrey, *Agave poselgerii*, Salm-dyck, and *Agave heteracantha*, Zucc., as synonymous names representing one and the same plant. . . . ."

This conclusion is evidently based on Engelmann (4) and Baker's (5) classification. In discussing *Agave lechuguilla* Torrey and its relationship, Mulford (6), states in conclusion:

"Though this plant certainly shows affinities with *Agave heteracantha* Zucc. and *Agave poselgerii* Salm-dyck, it differs from them in having a more stiffly suberect and one-sided habit and in never developing a broad pale band down the face of the leaf. The group to which all these and a number of related forms belong should receive careful study and comparison. Our plant may prove to be a variety."

She mentions also that the specific name was originally printed "*lecheguilla*" through an error and should be "*lechuguilla*." Michotte (1) refers to "*lechuguilla*" as "*Agave (littaea) multilineata*

Baker, *Agave heteracantha* Zuccar, c'est lechuguilla du Mexique," which classification is obviously erroneous, as Baker (5) specifically stated, "*Agave (littaea) multilineata* Baker is the same as *Agave heteracantha*, Hort. Angl. not Zucc." Rose (7) states that *Agave lechuguilla*

"has been confused with *A. heteracantha*, from which, although the two are closely related, it appears to be distinct. . . . Our herbarium seems to show at least four good species of the *heteracantha* group. . . . I should not hesitate to describe some of them as new if I understood what is really the type of *A. heteracantha* Zucc. and *A. poselgerii* Salm. I have the type of *A. lechuguilla* and have seen the description of *A. heteracantha*, but the latter answers to no specimens we have."

The material used in this investigation was obtained from Uvalde, Texas, and was collected in April, 1916. It consisted of the rootstocks and short stems, and in most cases the leaves were attached. In identifying this material as *Agave lechuguilla* Torrey, we have received the assistance of Prof. William Trelease, of the University of Illinois, and of Mr. L. H. Dewey and Mr. E. O. Wooton, of the United States Department of Agriculture, who have furnished a number of photographs, some of which show the general growth of the plant and the characteristic morphological structures (Fig. 1).

*Agave lechuguilla* is known to contain a substance which foams freely with water (8); in fact, the name "amole de lechuguilla" refers to this soapy character. The plant is valued, however, chiefly on account of the fibers which are abundant in the leaves and possess great strength and durability. The fiber is called Tampico ixtle and istle, or, according to more recent information, Tula ixtle (9). A saponin is found throughout the plant, in the rootstocks as well as in the leaves, which, after the fibers have been removed, are used locally as soap substitutes. It is said that shampoo mixtures are also prepared from the rootstocks of the agave. As far as we have been able to learn the saponin has not been isolated hitherto.

An almost colorless and practically ash-free saponin, which, however, could not be crystallized, has now been isolated from the alcoholic extract of the rootstock.

The hydrolysis of this saponin yielded a pro-sapogenin, a well crystallized end-sapogenin melting at 183.5°C., and two sugars. The end-sapogenin proved to be identical with a sapogenin previously obtained from a saponin from *Yucca filamentosa*. One of the sugars, which was isolated in crystalline form, was shown to be galactose; the other was identified as glucose by means of its phenyl- and para-bromophenylosazones and by its specific rotation.

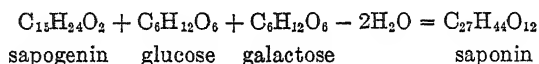


FIG. 1. Leaves of *Agave lechuguilla* Torrey showing the characteristic margin.

Although the saponin gave positive pentose tests with Bial's reagent and yielded furfural on distillation with concentrated hydrochloric acid, the authors could get no further evidence of the presence of a pentose. It is probable that the results of these tests were due to the galactose which is known to give such pentose reactions.

Numerous ultimate analyses were made of the saponin and its sapogenin and the molecular weight of these compounds was determined. Accurate analyses of the saponin were difficult to

obtain because of its hygroscopic character. The presence of a small quantity of moisture which could not be removed at 110°C. would, of course, tend to lower the carbon content. Heating to a higher temperature was not safe as it caused decomposition which was indicated by a darkening of the compound. The molecular weight determinations show that the composition of the saponin does not agree with Kobert's general formula (10) for saponins,  $C_nH_{2n-16}O_{28}$ . Even if Kobert's formula were altered to  $C_nH_{2n-8}O_{14}$ , the results obtained on the hydrolysis of the saponin cannot be made to agree with it. The sapogenin is a well defined crystalline compound with a sharp melting point and can readily be purified from the crude sapogenin by crystallization from alcohol. The results of the ultimate analysis and the molecular weight determination of this compound agree well with the formula  $C_{15}H_{24}O_2$ . It seems best, therefore, to deduce the formula of the saponin from its products of hydrolysis in the following manner:



#### EXPERIMENTAL.

##### *Histochemical Experiments.*

The saponin was found throughout the plant, in the roots, rootstocks, short axis, and leaves. It was most abundant in the basal parts of the plants, where the fibrovascular bundles were numerous and not especially fibrous and the tracheæ or tracheids formed an essential part of the bundles. A section through the rootstock or axis or even through the bases of the leaves, particularly in the fresh state, when moistened with concentrated sulfuric acid, revealed to the unaided eye the color changes which are characteristic of saponins. This reaction was especially distinct within the bundles, which at first became yellow and then slowly turned pink and reddish white.

Under the microscope the saponin masses were found most easily in the air-dried material. Here they appeared as amorphous, usually transparent, yellowish to yellow-brown films covering the walls or filling the cells of tracheæ and tracheids. They could also be found in the parenchymatous and sclerenchymatous

tissue surrounding these elements. In the fibers of the leaves the tracheæ form only a small part and are frequently scarcely visible without staining. Saponin may, however, be found in the tracheal veins, distributed rather abundantly in the parenchymatous tissue surrounding the fibers. From these observations it is believed that the saponin occurs in the sap of the cells. Since the saponin contains 2 molecules of sugar, it may be used as food material in the metabolism of the plant. The fact that the saponin is strongly hygroscopic suggests that it may also serve to retain moisture in the plant tissue during the long periods of draught prevailing in desert regions.

The histochemical studies were somewhat complicated by the fibrous character of the leaves and the serious decay of the root-stocks which usually occurs in grown agave plants. Since the saponin was easily soluble in water and in 95 per cent or more dilute alcohol, the plant material to be tested could not be immersed in these liquids. Unfortunately, lead acetate (neutral and basic), barium hydroxide, and cholesterol, which usually precipitate saponins, could not be used, as they caused no precipitation of saponin in this case. Ether, absolute alcohol, and olive oil were used for immersing the sections prepared in the experiments to locate the saponin. None of these liquids, however, were altogether satisfactory. Ether evaporates rapidly, absolute alcohol dissolves the saponin slowly, and olive oil is not readily removed from the tissue, making it difficult to complete the test.

The use of a suspension of blood corpuscles, recommended in a previous paper (11), was again tried, but gave satisfactory results only in the roots. Here calcium oxalate, although present in other parts of the plant, is absent; it also produces a strong hemolytic effect.

In addition to the hemolytic action upon blood corpuscles, the saponin masses were identified by their solubility in water, alcohol, phenol, and glacial acetic acid and by their insolubility in ether and chloroform. They also showed characteristic color changes with sulfuric acid, slowly changing from distinctly yellow to pink and reddish violet. The color change to pink or reddish violet could be observed almost immediately, if a mixture of equal parts of sulfuric acid and alcohol, or acetic anhydride followed by sulfuric acid, were used. A green color (not precipitate) was produced by

sulfuric acid containing less than 1 per cent of ferric chloride. If dried plant material is used, 70 or 80 per cent sulfuric acid is preferable to the more concentrated acid, which usually chars or destroys the tissue. The epidermis of the leaves, however, is so strongly suberized that the walls often resist the action of even concentrated sulfuric acid. The stomata imbedded in these epidermis cells at times gave a yellow color, turning to a distinct pink-red. Although this may have been due to sugars or gums which, in the presence of other substances, often give a color similar to that produced by saponins, it is possible also that some of the saponin enters these cells with the cell sap or is at times stored to retain or attract moisture. A 1 per cent potassium permanganate solution was also tried and was instantly decolorized by sections containing saponin. It is questionable, however, whether potassium permanganate which has been used as a reagent for substances of a saponin character is satisfactory for the microchemical detection and location.

The microchemical identification of the saponin by means of the products of hydrolysis has so far not been generally successful, although in sections exposed to the vapor of hydrochloric acid or left immersed for more than a day in 40 per cent sulfuric acid, the formation of crystals has, in a few instances, been observed within the saponin masses, which ordinarily showed no refraction of light.

#### *Isolation of the Saponin.*

From 1 to 10 kilos of the finely divided, air-dried rootstocks of *Agave lechuguilla* were extracted several times with hot 95 per cent alcohol, and the red alcoholic solution was evaporated to dryness on the steam bath after adding about 50 gm. of magnesium oxide for each kilo of the agave used. The residue was ground and extracted with hot absolute alcohol. The process was repeated until no more saponin could be obtained. On cooling the alcoholic solutions, a light-colored granular substance separated. This was filtered off by suction and washed once or twice with dry ether. The yield of crude saponin was about 9 per cent. The crude substance was again dissolved in hot absolute alcohol and the saponin allowed to come out by cooling. Repeating the process five times gave an almost white material which contained only a negligible trace of mineral matter.

The saponin remaining in the alcoholic filtrates was obtained by pouring the solutions into equal parts of ether, decanting, redissolving the precipitate in hot absolute alcohol, and allowing the substance to come out by cooling. By purifying several times in this way, more pure material was obtained. The same saponin was also isolated in the manner already described from the bases of the leaves, using only the white portion of the base.

The saponin was soluble in water, alcohol, phenol, and glacial acetic acid. Its aqueous solution foamed when shaken. When treated with concentrated sulfuric acid the color change from yellow to purple-red, generally produced by saponins, was obtained. Neutral lead acetate, basic lead acetate, and barium hydroxide did not precipitate it from either the aqueous or alcoholic solution. A 1 per cent solution of the saponin in water did not give a precipitate on adding a 1 per cent alcoholic solution of cholesterol. The saponin was very hygroscopic, and for analysis it was necessary to dry it carefully by gradually heating it to 110°C. in a vacuum oven until the weight was constant.

The following results were obtained on analyzing five preparations:

1. 0.1649 gm. substance:	0.3380 gm. CO <sub>2</sub>	and 0.1179 gm. H <sub>2</sub> O.
2. 0.1637 " "	0.3359 " "	" " 0.1178 " "
3. 0.1396 " "	0.2857 " "	" " 0.1008 " "
4. 0.2268 " "	0.4646 " "	" " 0.1587 " "
5. 0.0880 " "	0.1910 " "	" " 0.0635 " "

Preparation.....	1	2	3	4	5	Average.
C.....	55.90	55.96	55.82	55.87	56.13	55.93
H.....	8.00	8.05	8.08	7.83	8.08	8.01
O.....	36.10	36.99	36.10	36.30	35.79	36.06

*Molecular Weight in Phenol.*—0.5341 gm. of substance lowered the freezing point of 27.94 gm. of phenol, 0.229°.

$$M = \frac{7,500 \times 0.5341}{0.229 \times 27.94} = 626. \quad \text{Check 626.}$$

C<sub>27</sub>H<sub>44</sub>O<sub>12</sub>. Calculated. C 57.85, H 7.92, Mol. wt. 560.  
Found. " 55.93, " 8.01, " " 626.



## 342 Saponin from *Agave lechuguilla* Torrey

Further analyses carried out<sup>1</sup> with saponin, after further purification, did not yield materially different results:

Average analysis.		
	Before purification.	After purification.
	<i>per cent</i>	<i>per cent</i>
C	55.46	55.32
H	7.62	7.75

*Surface Tension of Saponin.*<sup>2</sup>—For the surface tension determination, the Morgan drop-weight method was used. At 37°C. the surface tension of a solution of 100 mg. per liter of Locke's<sup>3</sup> solution was 59.75 dynes per cm.

### *Biological Tests.*

*Hemolysis of Saponin.*<sup>2</sup>—For the hemolysis tests, blood was drawn from the heart of a Belgian hare into 0.9 per cent saline solution, to which a little potassium oxalate was added. The blood was then centrifugated and washed with Locke's solution. 2 drops of the residual corpuscles were mixed with 10 cc. of an aqueous saponin solution, 1 in 10,000, and kept at 37°C. Hemolysis took place in about 1 hour.

### *Toxicological Effect of the Saponin on Fish.*

In order to test the effect of the saponin on fish, experiments were made with minnows about 8 to 10 cm. long. The minnows were placed in dishes containing tap water and small quantities of the isolated saponin were added, the highest concentration used being 0.02 per cent. As soon as the saponin had been added the

<sup>1</sup> Analyses made by Mr. C. E. F. Gersdorff, Protein Investigation Laboratory, Bureau of Chemistry.

<sup>2</sup> Determined by Dr. E. H. Woodward of this Bureau.

<sup>3</sup> Locke's solution:

	<i>gm.</i>
NaCl.....	9.2
KCl.....	0.42
CaCl <sub>2</sub> .....	0.24
HNHCO <sub>3</sub> .....	0.15
H <sub>2</sub> O to.....	1 liter

fish became greatly excited and swam about rapidly, but soon calmed down and came to the surface of the water gasping for air. Bleeding in the vicinity of the gills and fins was observed. The fish lost their power of maintaining equilibrium and gradually turned over on their backs. After swimming in this position for sometime they died. The addition of cholesterol delayed and in some instances inhibited fatal action of the saponin (12). In order to compare the toxicity of the agave saponin with that of saponin obtained from other sources, experiments were made under similar conditions with a commercial sample of saponin from *Quillaja saponaria* Merck and a sample of saponin isolated by the authors from California soap-plant, *Chlorogalum pomeridianum* (13). The solution of the agave saponin caused the fish to turn completely over in from 3 to 5 minutes, while the same effect was produced by the quillaja saponin in 15 minutes and by the chlorogalum saponin in 2 hours. The agave saponin was, therefore, the most toxic of the three.

### *Hydrolysis of the Saponin.*

*Formation of a Pro-Sapogenin.*—50 gm. of saponin were dissolved in 300 cc. of 1 per cent sulfuric acid and the solution was heated on a steam bath for 18 hours. A tan-colored, amorphous pro-sapogenin settled out during the heating. This was thrown down in a compact mass by means of the centrifuge, the supernatant liquid was decanted, and the residue was washed until the washings were no longer acid to litmus. The pro-sapogenin was further hydrolyzed as described later.

*Identification of Glucose.*—The acid solution decanted from the pro-sapogenin as just described was combined with the wash water, and sulfuric acid was added until the concentration of the acid reached 5 per cent. This mixture was heated on a steam bath for 6 hours, during which time a tarry substance separated. The mother liquor was decanted and neutralized with barium carbonate, and the barium sulfate was filtered off. The filtrate was decolorized with animal charcoal and after filtering was evaporated down, giving a light yellow sirup. A solution of this sirup was strongly dextro-rotatory. The specific rotation of the sugar calculated as glucose was  $58.8^{\circ}$  to the right at  $20^{\circ}\text{C}$ . using sodium light. The specific rotation of glucose is  $53^{\circ}$  to the right, while

the specific rotation of galactose is  $81^\circ$  to the right. The rotation obtained was, therefore, somewhat high for glucose, but this is explained by the fact that the solution contained some galactose. Since mannose was absent and the solution was dextro-rotatory, the formation of phenylosazone (M.P.  $205^\circ$ ) and para-bromophenylosazone (M.P.  $222^\circ$ ) shows that glucose was one of the hydrolytic products.

*Formation of the End-Sapogenin.*—When the pro-sapogenin was boiled with 6 per cent sulfuric acid until foaming ceased, the end-sapogenin was obtained in granular form. This was filtered off and was freed from acid by means of water. It was then dissolved in hot dilute alcohol, from which a white crystalline substance separated on cooling. Upon concentration of the mother liquor the same substance formed plates and prisms in addition to needles. The plates were undoubtedly somewhat impure, melting at  $180^\circ$ . The crystal mass was further purified by recrystallization from 95 per cent alcohol. In this manner fine acicular crystals or prisms, melting at  $183.5^\circ\text{C}$ ., were obtained. The substance was insoluble in water, but soluble in alcohol, acetone, benzene, and phenol. It was insoluble in dilute acids and alkali, but dissolved easily in concentrated acids. The sapogenin appears to be a very stable compound. When heated at  $100^\circ\text{C}$ . for 6 hours in an alcoholic solution of potassium hydroxide, saturated in the cold, it was recovered unaltered. A mixture of potassium dichromate and 50 per cent sulfuric acid also has no action on it.

The analyses of this compound gave the following results:

0.2805 gm. substance:	0.2560 gm. $\text{H}_2\text{O}$	and 0.7827 gm. $\text{CO}_2$ .
0.2278 " "	0.2154 " "	0.6362 " "
0.1811 " "	0.1650 " "	0.5041 " "
0.1609 " "	0.1505 " "	0.4487 " "

	Preparation 1.	Check.	Preparation 2.	Check.	Average.
C	76.10	76.10	76.17	76.10	76.14
H	10.21	10.21	10.43	10.58	10.32

*Molecular Weight in Phenol.*—0.1980 gm. substance lowered the freezing point of 29.30 gm. of phenol  $0.206^\circ$ .

$$M = \frac{7,500 \times 0.1980}{29.35 \times 0.206} = 246. \quad \text{Check } \begin{cases} 245 \\ 246 \\ 245 \end{cases}$$

$C_{15}H_{24}O_2$ . Calculated. C 76.27, H 10.17, Mol. wt. 236.  
 Found. " 76.14, " 10.32, " " 246.

From its solubility, molecular weight, melting point, and crystallographic and optical properties, the sapogenin appears to be identical with the yucca sapogenin.

*Crystallographic and Optical Properties of Sapogenin from Agave.*<sup>4</sup>

*Crystallography.*—System, monoclinic; axial ratio  $a:b:c = 1.515 : 1 : 2.006$ , all  $\pm 0.001$ ; axial angle  $\beta = 85^\circ 45' \pm 3'$ . Forms:  $c$  (001),  $a$  (100),  $m$  (110),  $d$  (101), and  $p$  (111) developed in the order named. Habit mostly tabular on  $c$  and elongated on axis  $b$ , thus agreeing with Fedorov's rule as to the relation between axial ratio and habit. Faces somewhat rounded, often slightly concave; signals accordingly not particularly well reflected.

ANGLE TABLE.

No.	Form.	Symbols.		Development.	$\varphi$	$\rho$
		Gdt.	Mill.			
1	c	0	001	Dominant.	$90^\circ 00'$	$4^\circ 15'$
2	a	$\infty 0$	100	Prominent.	90 00	90 00
3	m	$\infty$	110	"	33 30	90 00
4	d	10	101	Narrow.	90 00	54 30
5	p	1	111	{ Small.	$35 \pm$	$68 \pm$
				{ Calculated.	34 57	67 47

*Optical Properties.*—Large crystals show parallel extinction and optic axial plane coinciding with plane of symmetry, one optic axis emerging obliquely through base. When crushed yields angular fragments; attacked but slowly by immersion oils; refractive indices (D).  $\alpha = 1.535$ ,  $\beta = 1.550$ ,  $\gamma = 1.570$ ,  $\gamma - \alpha = 0.035$ , all  $\pm 0.005$ ; means are usually shown. Double refraction strong, colors being first to second order on small grains; extinction and elongation indeterminate; class biaxial;  $2E$  very large; sign +; dispersion distinct.

*Isolation of Galactose.*—The acid solution obtained from hydrolysis of the pro-sapogenin was neutralized with barium carbonate. After filtering off the barium sulfate, the filtrate was clarified with animal charcoal and evaporated to a sirup. This was dissolved in cold 95 per cent alcohol, and absolute alcohol was added until

<sup>4</sup>Determined by Dr. E. T. Wherry, Crystallographer, Bureau of Chemistry.

no more gummy precipitate was produced. After standing over night the supernatant liquid was decanted. This was mixed with an equal volume of anhydrous ether and the precipitate was allowed to settle. The mother liquor was decanted and the white residue was crystallized from about 98 per cent alcohol. Small acicular crystals melting at 165°C. were obtained. When mixed with pure galactose, there was no lowering of the melting point. Evaporation of the sugar with nitric acid gave mucic acid. The phenylosazone of the sugar melted at 197°C. and the para-bromophenylhydrazone melted at 168°C. There is, therefore, no doubt that this sugar was galactose.

#### SUMMARY.

1. A saponin not hitherto described was isolated from the rootstock with attached roots and short overground axis, and the bases of the leaves of *Agave lechuguilla*. The results of the ultimate analyses, the molecular weight determination in phenol, the nature of the products of hydrolysis of the saponin, and especially the results of the analysis of the sapogenin suggested the formula  $C_{27}H_{44}O_{12}$ . The saponin was soluble in water, alcohol, and phenol. Lead acetate, lead subacetate, and barium hydroxide did not precipitate it from either the aqueous or alcoholic solutions, nor did cholesterol form an insoluble compound. The aqueous solution containing 100 mg. of saponin per liter hemolyzed rabbit's blood in about 1 hour at 37°C. Its surface tension at 37°C. in Locke's solution was 59.75 dynes per cm.

2. Experiments with fish indicated that the saponin is more toxic than that from the common soapbark, *Quillaja saponaria*, or from the California soap-plant, *Chlorogalum pomeridianum*.

3. The saponin occurs in the cell sap and may be located in the air-dried plant in the fibrovascular bundles or veins of the roots, rootstock, axis, and leaves. The rootstock and roots, apparently containing the largest amounts, yielded in an air-dried state about 9 per cent of crude saponin.

4. The hydrolysis of this saponin yielded glucose and a pro-sapogenin.

5. The hydrolysis of the pro-sapogenin gave galactose and an end-sapogenin.

6. The end-sapogenin, melting at  $183.5^{\circ}\text{C}.$ , and to which the formula  $\text{C}_{15}\text{H}_{24}\text{O}_2$  is assigned, proved to be identical with a sapogenin previously obtained from a saponin from *Yucca filamentosa*. Its crystallographic optical properties were determined.

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## A COLORIMETRIC METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF MAGNESIUM.

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Colorimetric methods for the determination of phosphorus in blood and urine have recently been published by Bell and Doisy,<sup>1</sup> based upon the selective reduction of phosphomolybdic acid by hydroquinone in the presence of excess ammonium molybdate.

In the method here described use is made of the principle of this method for the determination of the phosphorus content of precipitated  $\text{MgNH}_4\text{PO}_4$ . The method is obviously applicable to any substance from which  $\text{MgNH}_4\text{PO}_4$  may be precipitated. At present it is being used chiefly for the determination of magnesium in conjunction with calcium and other inorganic elements in the trichloroacetic acid filtrates from citrated blood plasma.

*Procedure.*—By means of a pipette, transfer a measured volume of the plasma into a small flask, dilute with 3 volumes of water and 1 volume of 20 per cent trichloroacetic acid, mix by shaking, and pour onto an ashless filter. Transfer 15 cc. of the filtrate into a 25 cc. Pyrex test-tube. Add 1.5 cc. of the potassium acetate solution and 2 cc. of the ammonium oxalate solution; rub up and down inside the tube with a rubber-tipped rod until the  $\text{CaC}_2\text{O}_4$  seems to be completely precipitated and rinse off the rod with a little water. Heat the tube 15 minutes in a boiling water bath, and after cooling to room temperature centrifugate for 5 minutes at about 2,000 R.P.M. Pour the supernatant liquid from the calcium precipitate into a 50 cc. centrifuge tube. Add 1 cc. of the ammonium phosphate solution and 5 cc. of concentrated ammonia and rub up and down inside the tubes with a rubber-tipped rod until precipitation seems to be complete. After standing 3 or 4 hours,

<sup>1</sup> Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.



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centrifugate for 10 minutes at about 1,500 R.P.M. and pour off the supernatant liquid. Fill the tubes about half full with the ammoniacal alcohol wash solution, and rinse down the sides of the tube carefully with a jet of the same solution from a wash bottle. Centrifugate 5 minutes and pour off the wash fluid, wash a second time in the same manner. Dissolve the precipitate in 5 cc. of normal  $\text{H}_2\text{SO}_4$ , add 1 cc. of the molybdate solution and 1 cc. of the hydroquinone solution, and dilute with water up to the graduation mark at 20 cc. Prepare a standard at the same time containing 5 cc. of the standard magnesium solution and the same amounts of  $\text{H}_2\text{SO}_4$ , molybdate, and hydroquinone. Mix the solutions and after 5 minutes compare in a colorimeter.

### *Solutions Used.*

*Standard magnesium solution*, contains 0.1413 gm. of  $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$  per liter in 0.01 N  $\text{H}_2\text{SO}_4$  and is preserved by the addition of 2 cc. of chloroform. 5 cc. of this solution are equivalent to 0.07 mg. of magnesium which is about the amount found in 3 cc. of plasma.

*Molybdate solution*, contains 5 per cent ammonium molybdate in N  $\text{H}_2\text{SO}_4$ .

*Hydroquinone solution*, contains 2 per cent hydroquinone.

*Potassium acetate solution*, prepared as follows: Dissolve 125 gm. of  $\text{K}_2\text{CO}_3$  in as little water as possible and let stand over night. Filter and neutralize with 100 cc. of glacial acetic acid, and dilute to 500 cc. with water. This solution was found to be free from calcium and magnesium. All available samples of sodium acetate were found to contain calcium or magnesium.

*Ammonium phosphate*, 2 per cent solution of  $(\text{NH}_4)_2\text{HPO}_4$ , preserved with chloroform.

*Ammonium oxalate*, a saturated solution.

*Ammoniacal alcohol*, contains 200 cc. of 95 per cent alcohol and 50 cc. of concentrated ammonia per liter.

To show that the color obtained was proportional to the amount of  $\text{MgNH}_4\text{PO}_4$ , various amounts from 5 to 20 cc. of the standard solution were transferred to 25 cc. volumetric flasks. To each were added 2 cc. of the molybdate solution, 2 cc. of the hydroquinone solution, and water up to the mark. After 5 minutes the color in each flask was compared with the one containing 12 cc. of the standard solution. The readings in Table I were obtained.

To test the complete recovery of magnesium from trichloroacetic acid filtrates, measured amounts of the standard solution were transferred to 50 cc. centrifuge tubes. To each were added 3 cc. of 20 per cent trichloroacetic acid, 2 cc. of the ammonium oxalate solution, 1 cc. of the ammonium phosphate solution, 1.5 cc. of the potassium acetate solution, enough water to bring the volume

TABLE I.

*Showing the Proportionality between the Amount of Standard  $MgNH_4PO_4$  Solution Taken and the Intensity of Color Produced.*

Amount of standard solution taken.	Reading obtained on comparing with 12 cc.	Theoretical reading.
cc.		
5	10:23.2*	10:24.0
10	20:24.0	20:24.0
12	20:20.0	20:20.0
15	20:16.0	20:16.0
20	20:12.3	20:12.0

\* Solution with 12 cc. of standard at 10 mm.; that with 5 cc. at 23.2 mm.

TABLE II.

*Showing the Recovery of Mg from Trichloroacetic Acid.*

Amount of Mg taken.	Mg recovered.
mg.	per cent
0.10	102
0.10	98
0.10	100
0.10	102
0.15	100
0.15	102
0.03	99
0.03	104

up to about 20 cc., and finally 5 cc. of concentrated ammonia. The procedure described above was then followed and comparison made with a standard containing the amount of magnesium solution taken for the test.

Table II shows the recovery to be satisfactory.

The precipitate as it is obtained in the determination, packs well enough in ordinary round bottomed centrifuge tubes, so that supernatant fluids may be completely poured off with little or no

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loss of magnesium. For this reason only one or two washings are required to remove the soluble phosphates which, if not completely removed, would make the results high. Several determinations on 10 cc. portions of the standard magnesium solution have shown that the results after one washing are only from 2 to 6 per cent high and that more than two washings are superfluous.

Certain errors are evident in the determination as described. Magnesium is determined on the supernatant liquid poured off from the calcium precipitate. If the volume poured off is about 20 cc. and the amount retained in the tube with the calcium precipitate is about 0.2 cc. then an error of approximately 1 per cent is introduced. When the wash fluids are poured off from the  $\text{MgNH}_4\text{PO}_4$  precipitate small losses are also sustained. Another

TABLE III.  
*Recovery of Mg after Removal of Ca.*

Reading.	Mg recovered.
	<i>per cent</i>
20:20.5	97.5
20:20.4	98.0
20:20.8	96.0
20:20.0	100.0
20:20.4	98.0
20:19.8	101.0

error of about 1 per cent may be incurred when diluting to the 20 cc. graduation mark on the 50 cc. centrifuge tube.

The experiments below indicate that a total error of less than 4 per cent may be expected when working with such amounts as are found in 3 cc. of blood plasma. Magnesium determinations were made on 15 cc. portions of a known solution, each containing 0.1 mg. of magnesium, 0.5 mg. of calcium, and 3 cc. of 20 per cent trichloroacetic acid. All the steps in the method were carried out as described and the readings in Table II obtained on comparison with a standard containing 0.1 mg. of Mg set at 20 mm.

Very small amounts of calcium oxalate are, of course, poured off with the supernatant liquid from the calcium precipitate, this, however, introduces no error into the magnesium determination, as the calcium oxalate is not changed to the more soluble phosphate. In fact the magnesium determinations on blood plasma or urine

are the same if made on the combined  $\text{CaC}_2\text{O}_4$  and  $\text{MgNH}_4\text{PO}_4$  as they are on the latter alone. Larger amounts of oxalates retard the formation of the color and make the results low.

If the calcium is precipitated in the presence of too much acid, the separation of  $\text{CaC}_2\text{O}_4$  is incomplete. In this case Ca in solution would be present when the Mg is precipitated and the condition would be more favorable for the formation of some calcium phosphate as well as oxalate. But it was found that when 0.1 mg. of Mg and 0.5 mg. of Ca were treated with 1 cc. of the ammonium phosphate solution, and 2 cc. of the ammonium oxalate solution with sufficient acid present to prevent any precipitation, then made strongly ammoniacal, that the calcium was all precipitated as

TABLE IV.

*Recovery of Mg after Precipitation of Ca at pH 4.0 to 5.0, from a Solution Containing a Large Amount of Phosphate.\**

pH 4.0		pH 5±	
Mg taken.	Mg recovered.	Mg taken.	Mg recovered.
mg.	mg.	mg.	mg.
40.0	39.39	40.0	39.50
40.0	39.44	40.0	39.44

\* The Ca determinations were all approximately 100 per cent.

oxalate and the magnesium as phosphate. The color obtained from the combined precipitates matched a standard equivalent to 0.1 mg. of magnesium.

The trichloroacetic acid filtrates were neutralized in the manner described in preference to the technique of McCrudden<sup>2</sup> to minimize the salts in the calcium filtrate. McCrudden found that large amounts of salts in the calcium filtrates made the subsequent magnesium determinations low. When 3 cc. of 20 per cent trichloroacetic acid or 15 cc. of the plasma filtrate are neutralized with 1.5 cc. of the potassium acetate solution using a drop of methyl orange as indicator, the color is seen to change from red to yellow with a slight tinge of brown, corresponding to an acidity of pH 4 to 5±. To test the separation of Ca and Mg at the extremes of this range, in the presence of phosphates, additional de-

<sup>2</sup> McCrudden, F. H., *J. Biol. Chem.*, 1909-10, vii, 83.

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terminations were made on 100 cc. portions of a solution containing 0.04 per cent calcium, 0.04 per cent magnesium, and 1 per cent ammonium phosphate. Methyl orange was added as indicator and potassium acetate added in one set until the color matched that of 0.0001 N HCl containing the same amount of methyl orange. In the other set potassium acetate was added until the color changed to yellow.  $\text{CaC}_2\text{O}_4$  was precipitated with excess of saturated  $(\text{NH}_4)_2\text{C}_2\text{O}_4$  solution and magnesium determined on the filtrates by gravimetric procedure.<sup>3</sup>

TABLE V.  
*Mg in 100 Cc. of Urine.*

Colorimetric.	Gravimetric.
<i>mg.</i>	<i>mg.</i>
3.38	3.28
3.43	3.38

TABLE VI.  
*Mg Determinations on Blood Plasma.*

Character of plasma.	Mg per 100 cc.
	<i>mg.</i>
Human, early pregnancy.....	2.23
“ “ “ .....	2.50
“ “ “ .....	2.35
“ “ “ .....	2.32
“ normal male.....	2.48
Beef.....	2.20

Table IV shows the recovery of magnesium to be very nearly complete.

The following comparison was made with gravimetric procedure on a normal urine. The urine was made acid with a few drops of concentrated  $\text{H}_2\text{SO}_4$  and filtered. Duplicate samples of 5 cc. each were pipetted into 50 cc. centrifuge tubes and diluted to about 20 cc., treated with 2 cc. of saturated ammonium oxalate, 2 cc. of the ammonium phosphate solution, and 5 cc. of concentrated ammonia. Precipitation was accelerated by rubbing and after standing about 4 hours the combined precipitates of  $\text{CaC}_2\text{O}_4$  and  $\text{MgNH}_4$ -

<sup>3</sup> Jones, W., *J. Biol. Chem.*, 1916, xxv, 87.

$\text{PO}_4$  were centrifuged and washed and compared with a standard containing 0.20 mg. of Mg.

Gravimetric determinations were made on 100 cc. samples of the same urine. Ca was precipitated at  $\text{pH } 4.5 \pm$  with excess ammonium oxalate, the filtrates were evaporated to dryness and ignited in platinum dishes, and Mg was determined on the residue according to the procedure of Jones.<sup>3</sup> The results are shown in Table V.

The few preliminary determinations on normal plasmas tabulated in Table VI indicate the maintenance of a rather constant concentration of magnesium.



## STUDIES ON THE DIGESTIBILITY OF PROTEINS IN VITRO.

### III. ON THE CHEMICAL NATURE OF THE NUTRITIONAL DEFICIENCIES OF ARACHIN.\*

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In discussing the results of a previous investigation (1) we suggested that an explanation of the effect of partial indigestibility upon the growth-promoting properties of a protein might be found in the presence of some one or more of the essential amino-acids in combinations which, like certain polypeptides studied by Fischer and Abderhalden (2), resist the hydrolytic action of digestive enzymes by reason of the order in which their component amino-acid residues are united. The authors cited found the same amino-acids which constituted their indigestible polypeptides to be capable of forming readily hydrolyzable compounds when united in a different order.

The presence of such a difficultly digestible complex in the molecule of arachin, the principal protein of the peanut, *Arachis hypogaea*, has recently been suggested by Barnet Sure (3), in connection with a study of the biological inadequacy of this protein. The high nutritive value of peanut press-cake preparations (4, 5, 6) this author explains as due to the presence in the total peanut protein mixture of some source of the amino-acids which arachin, by reason of its resistance to the action of proteoclastic enzymes, does not supply. Amino-acid deficiencies could not be demonstrated by supplementing with proline, with the amino-acids of the leucine fraction, with cystine or tryptophane, nor with all these combined (3). A chemical analysis (7) had already shown arginine, histidine, and lysine

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to be present in such proportions as to make it appear very unlikely that a deficiency (in the ordinary, quantitative sense) in any one of them could be the limiting factor. Sure concludes that until further evidence, based upon specific amino-acid additions, shall have been secured, the nutritional failure of arachin must tentatively be ascribed to incomplete digestibility resulting from the order in which the amino-acid nuclei are combined.

The purposes of the experiments described in the present paper were: (a) to ascertain if incomplete digestibility of arachin could be demonstrated by experiments *in vitro*; (b) if such an incomplete digestibility were found to exist, to determine if it could be remedied by any means not productive of a far reaching hydrolysis; and (c) to secure, by means of a chemical study of partial degradation products of arachin, some evidence as to the identity of the amino-acids which are so combined in arachin as to be difficultly separable by proteoclastic enzymes.

With respect to the first of these points, our results support the tentative conclusion drawn from the preceding animal experiments. Arachin was found to be less digestible *in vitro* than any biologically available protein thus far tested under the same conditions. A test of the second point gave a result quite contrary to that which our previous experiences (1, 8) had led us to expect. The digestibility of arachin *in vitro* was not improved by heating with water, even when this was done under a pressure of 15 pounds and continued for 1 hour. The experiments on the third point yielded very interesting results. By acting upon arachin with 0.10 N sodium hydroxide at 80° a partial cleavage product was obtained which amounted to about one-third of the original protein. This derivative was very difficultly digestible *in vitro*; and on analysis it was found to have retained a disproportionate amount of the basic nitrogen contained in the arachin from which it was prepared.

The preparation analyzed constituted 33.8 per cent of the original protein. Of the 1.88 per cent of histidine contained in arachin, 1.31 per cent, about two-thirds of the total, was found in this preparation. Of 13.51 per cent of arginine and 1.14 per cent of cystine indicated by the analysis of arachin, 4.55 and 0.37 per cent, about one-third in each case, were found in the resistant fraction, while of 4.98 per cent of lysine our derivative contained 1.95 per cent, about two-fifths of the total quantity.

On the basis of this information, feeding experiments are now being carried on in this laboratory with the purpose of determin-

ing: (a) if arachin may be supplemented effectively with any combination of the hexone bases and cystine; and (b) to ascertain whether or not the products of a complete hydrolysis of arachin, supplemented with the amino-acids known to be destroyed by the hydrolyst employed, are capable of supporting normal growth.

#### EXPERIMENTAL.

##### *Estimation of Relative Digestibility in Vitro.*

*Outline of Method.*—The procedure in all the estimations of relative digestibility made in this investigation was a slightly modified form of that described by Waterman and Johns (8) in the first paper of this series. It has been treated in detail in the first and second papers (1, 8). Briefly, it was as follows: Duplicate samples, approximately 0.500 gm., in the form of 100 mesh powder, of each of the proteins between which a direct comparison was desired were digested for  $1\frac{1}{2}$  hours at  $37^\circ$  in 50 cc. of 0.10 N hydrochloric acid containing 0.1 per cent of pepsin. Then the reaction mixtures were neutralized by the addition to each of 5 cc. of N sodium hydroxide and treated each with 5 cc. of a 6 per cent solution of trypsin in 0.10 N sodium hydroxide. The resulting 60 cc. of approximately 1/120 N sodium hydroxide containing 0.5 per cent of trypsin was allowed to act upon the samples for a further  $2\frac{1}{2}$  hours at  $37^\circ$ . After the digestion the activity of the enzymes was destroyed by heating at 75 to  $80^\circ$  on a steam bath for 5 minutes. The solutions were then filtered, and the amino nitrogen resulting from the digestion was determined by means of Van Slyke's amino nitrogen apparatus.

The results were calculated on the basis of the amino nitrogen of complete hydrolysis *minus* the free amino nitrogen (one-half the lysine nitrogen) of the protein. The formula is that given in our second paper on this subject:

$$\text{Per cent of digested nitrogen, DN} = \frac{N_d - N_b}{N_a} \times 100.$$

The terms of the equation are defined at the heads of the columns containing the corresponding data in the tables.

*Digestibility in Vitro of Arachin,<sup>1</sup> as Compared with That of Proteins of Known Digestibility.*—To determine if arachin was difficultly digestible under the conditions of our experiments, it

<sup>1</sup> The ordinary preparation of arachin, made according to the direction of Johns and Jones (11). This material was prepared by Mr. C. E. F. Gersdorff of this laboratory.

was compared directly with casein and with cooked phaseolin. The latter two proteins were chosen as standards both by reason of the fact that they have been shown to be sufficiently digestible to be assimilated by growing animals, and because the behavior of both of them in a test of this kind was familiar to us from previous experience.

In each case the digestibility *in vitro* of the arachin was found to be considerably less than that of the control protein (Table I).

TABLE I.  
*Digestibility in Vitro of Arachin as Compared with That of Proteins of Known Digestibility.*

Preparation.	Amount of sample (protein).	Combined amino N in sample (N <sub>a</sub> ).	Amino N after digestion (N <sub>d</sub> ).	Amino N of blank digestion (N <sub>b</sub> ).	Digestion N, calculated on basis of N <sub>a</sub> .
	mg.	mg.	mg.	mg.	per cent
Raw arachin.....1	500.8	63.61	49.99	19.20	48.4
2	501.8	63.74	49.67	19.20	47.8
Average.....					48.1
Casein.....1	501.2	49.37	49.32	19.20	61.0
2	501.3	49.38	49.66	19.20	61.7
Average.....					61.4
Raw arachin.....3	502.1	63.77	49.31	18.18	48.8
4	502.2	63.79	49.31	18.18	48.8
Average.....					48.8
Cooked phaseolin.....1	501.8	52.09	48.60	18.18	58.4
2	502.5	52.16	48.94	18.18	59.0
Average.....					58.7

Also, the qualitative indications noted in our previous work with difficultly digestible proteins were again observed in the case of arachin, while the control proteins as before, did not show these characteristics.

*The Digestibility in Vitro of Various Cooked Preparations of Arachin, and of a Preparation Made<sup>2</sup> Without Dehydration by*

<sup>2</sup> Preparation D, with the elementary analyses recorded in this paper, was made by Mr. S. Phillips of this laboratory.

*Alcohol*.—Previous experience had led us to believe that difficultly digestible vegetable proteins might be rendered biologically available (9, 10) and correspondingly readily digestible *in vitro* (1, 8) by heating with water. Preparations were accordingly made by suspending ordinary raw arachin in twenty times its

TABLE II.  
*Comparative Digestibility in Vitro of Raw and Cooked Arachin.*

Preparation.	Amount of sample (protein).	Combined amino N in sample ( $N_a$ ).	Amino N after digestion ( $N_d$ ).	Amino N of blank digestion ( $N_b$ ).	Digestion $N$ , calculated on basis of $N_a$ .
	mg.	mg.	mg.	mg.	per cent
Raw arachin.....5	502.3	63.80	48.15	17.60	47.9
6	503.8	63.99	48.48	17.60	48.3
Average.....					48.1
Cooked arachin A.....1	503.3	63.58	50.87	17.60	50.7
2	502.4	65.46	51.14	17.60	51.2
Average.....					51.0
Raw arachin.....7	502.1	63.77	51.12	19.22	50.0
8	501.7	63.72	50.96	19.22	49.8
Average.....					49.9
Cooked arachin B.....1	501.0	65.74	51.96	19.22	49.8
2	501.3	65.77	51.96	19.22	49.8
Average.....					49.8
Raw arachin.....9	501.5	63.70	50.97	19.28	49.7
Cooked arachin C.....1	500.5	63.57	51.05	19.28	50.0
2	501.4	63.69	51.41	19.28	50.5
Average.....					50.3

weight of distilled water and boiling for  $\frac{3}{4}$  hour (Preparation A), for  $2\frac{1}{2}$  hours (Preparation B), and for 1 hour under 15 pounds steam pressure (Preparation C). In no case, however, did the cooked arachin show a digestibility significantly superior to that of the ordinary, raw preparation (Table II).

In view of the high nutritional value of peanut meal, it seemed not impossible that the arachin preparations used in the feeding experiments and in our digestion experiments might have suffered some change in the process of isolation which would render indigestible an originally readily digestible protein. The only treatments involved in the preparation of arachin by the method of Johns and Jones (11) which seemed at all likely to have such an effect upon the protein were the dehydration with absolute alcohol and the subsequent dry heating at 110° in a drying oven. Preparation D was therefore made in accordance with the direction of Johns and Jones with the exception that neither alcohol nor ether were used in drying it, while the dry heating at 110° was replaced by treatment *in vacuo* at 40°.

The digestibility *in vitro* of this preparation was no greater than that of the raw and the cooked protein, however (Table III). Our results thus far, then, supported the theory that arachin, in itself incompletely digestible, is supplemented by some other source of amino-acids in peanut meal. Our next experiments were concerned with the digestibility and the chemical composition of partial breakdown products.

*Experiments with the Products of a Partial Hydrolysis of Arachin by Dilute Alkali.*—An experiment of Bateman (12) has shown that egg albumin, in its natural state difficultly assimilable, was rendered much more readily digestible by treatment with dilute alkali. We therefore subjected arachin to a similar treatment, after finding that neither cooking nor the elimination of alcohol dehydration and dry heating enabled us to produce a readily digestible preparation; for we wished to secure, if possible, a preparation sufficiently digestible to permit of a conclusive animal experiment to determine whether or not the amino-acid composition of arachin is adequate for normal growth.

Preparation E was made by dissolving arachin in 20 volumes of 0.1 N sodium hydroxide and heating the solution on a water bath at about 80° for 1 hour. The protein was precipitated by neutralizing with very dilute hydrochloric acid. The precipitate was filtered off, washed with warm distilled water, dehydrated by suspension in absolute alcohol, washed with ether, and dried under diminished pressure at 100°. Preparation F was made in an entirely similar manner.

Instead of producing the result noted by Bateman in the case of egg white, however, the alkali, under the conditions which we used, effected a partial hydrolysis of apparently considerable extent. Notable quantities of ammonia were given off during the warming on the water bath; hydrogen sulfide was evolved when the solution was acidified to precipitate the protein, indicating that a sulfur-bearing nucleus had been attacked; and from 65 to

TABLE III.

*Digestibility in Vitro of Arachin (Preparation D) Dried without Using Alcohol or Ether, or Heating at 110°, as Compared with That of Arachin Isolated in the Usual Way.*

Preparation.	Amount of sample (protein).	Combined amino N in sample (N <sub>a</sub> ).	Amino N after digestion (N <sub>d</sub> ).	Amino N of blank digestion (N <sub>b</sub> ).	Digestion N, calculated on basis of N <sub>a</sub> .
	mg.	mg.	mg.	mg.	per cent
Arachin D.....1	501.5	61.73	48.68	18.78	48.4
2	501.2	61.70	48.35	18.78	47.9
Average.....					48.2
Raw arachin.....10	502.2	63.79	49.66	18.67	48.6
11	501.5	63.70	49.39	18.67	48.2
Average.....					48.4
Arachin D.....3	501.2	61.70	49.23	18.67	49.5
4	501.7	61.76	49.72	18.67	50.3
Average.....					49.9

Final averages:	per cent
Arachin D.....	49.1
Ordinary raw arachin.....	48.4

80 per cent of the arachin treated was converted into products which could not be precipitated by dilute acid. The yield of Preparation F was 33.8 per cent. The product in this case was evidently not the slightly modified, probably internally rearranged but otherwise nearly intact, original protein, such as Bateman obtained in treating egg white. Rather, it must have consisted of those combinations in the arachin molecule which offer a relatively strong resistance to the hydrolytic action of

alkalies. This view is supported by the extraordinarily low digestibility of these preparations *in vitro* (Table IV). This latter condition may have been due in part to racemization, however.

It had been shown by Northrop (13) that alkali (sodium hydroxide) attacks largely the same linkages in the protein molecule as do pepsin and trypsin. It seemed therefore probable that an analysis of the alkali-resistant residue in arachin would yield interesting information, and that it might, indeed, furnish sugges-

TABLE IV.

*Digestibility in Vitro of the Unhydrolyzed Residue from the Treatment of Arachin with 0.10 N Sodium Hydroxide.*

Preparation.	Amount of sample (protein).	Combined amino N in sample ( $N_a$ ).	Amino N after digestion ( $N_d$ ).	Amino N of blank digestion ( $N_b$ ).	Digestion N, calculated on basis of $N_a$ .
	mg.	mg.	mg.	mg.	per cent
Preparation E..... 1	500.0	63.70	34.82	19.20	24.5
2	500.0	63.70	34.75	19.20	24.4
Average.....					24.5
Ordinary arachin.... 13	500.9	63.62	49.94	19.20	48.4
14	500.7	63.60	48.75	19.20	46.5
Average.....					47.5
Preparation F..... 1	500.0	63.70	36.75	19.54	27.0
2	500.0	63.70	36.09	19.54	26.0
Average.....					26.5

tions for the supplementation or other feeding experiments, to secure a basis for which this investigation was made. For this purpose the alkali product had the advantage that it was not contaminated, as would be the residue remaining after the action of pepsin and trypsin, with the protein contained in the enzyme preparations. The Van Slyke method (14) was used, since it gives values for the essential amino-acids except tryptophane. The result of this analysis, compared with the figures obtained by Johns and Jones (7), using the same method, for ordinary arachin, are shown in Tables V and VI.

TABLE V.

*Analysis by Van Slyke's Method of the Residue (Preparation F) Remaining After the Treatment of Arachin with Dilute Alkali, Compared with That of Ordinary Arachin.\**

	I	II	I	II	Average.	Ordinary arachin,* average.
	gm.	gm.	per cent	per cent	per cent	per cent
Amide N.....	0.0388	0.0378	7.95	7.75	7.85	11.81
Humin N.....	0.0026	0.0044	0.53	0.82	0.68	0.57
Cystine N.....	0.0034	0.0037	0.70	0.76	0.73	0.74
Arginine N.....	0.1134	0.1134	23.25	23.25	23.25	23.77
Histidine N.....	0.0300	0.0294	6.15	6.03	6.09	2.78
Lysine N.....	0.0318	0.0306	6.52	6.27	6.40	5.22
Amino N, filtrate.....	0.2601	0.2569	53.33	52.66	53.00	53.30
Non-amino N, filtrate.....	0.0103	0.0154	1.93	3.16	2.55	1.65
Total nitrogen recovered....	0.4904	0.4916	100.36	100.70	100.55	100.27

\* Johns and Jones (7).

TABLE VI.

*Percentages of the Basic Amino-Acids in the Residue (Preparation F), Remaining After the Treatment of Arachin with Dilute Alkali, as Compared with Those of Ordinary Arachin.*

Amino-acid	I	II	Average.	Ordinary arachin (7), average.
	per cent	per cent	per cent	per cent
Cystine.....	1.12	1.03	1.08	1.14
Arginine.....	12.47	12.47	12.47	13.51
Histidine.....	3.92	3.84	3.88	1.88
Lysine.....	5.87	5.65	5.76	4.98

The facts thus far brought out suggest some interesting possibilities. It seems best, however, to defer any attempt to formulate an interpretation of them until the results of the feeding experiments become available. "From the animal to the laboratory, and from the laboratory back to the animal is the logical order in such researches" (Hopkins, 15).

## SUMMARY.

Arachin is less readily digestible *in vitro* by pepsin and trypsin under the experimental conditions described than are any biologi-



cally available proteins which have thus far been tested by the same method. In direct comparison in the same experiment, arachin gave the figure 48 per cent as against 58 per cent for *cooked* phaseolin, and 61 per cent for casein.

The digestibility *in vitro* of arachin was not appreciably increased by boiling with water at ordinary pressure, or by steaming at 15 pounds pressure. The treatments involved in the isolation of arachin are apparently not responsible for the relatively low digestibility *in vitro*.

The action of hot, dilute sodium hydroxide upon arachin produced a partial cleavage derivative amounting to about one-third of the arachin from which it was prepared. This derivative contained about two-thirds of the total histidine, about one-third of the total arginine and of the total cystine, and about two-fifths of the total lysine of the original arachin. This partial cleavage product was very difficultly digestible *in vitro*.

The work of Northrop (13) is cited as showing that the action of hot, dilute alkalis is similar in some cases to that of pepsin and trypsin; and projected experiments with animals are outlined, the purpose of which is to determine if the basic amino-acids, found to be present in relatively considerable quantities in the alkali-resistant fraction of arachin, play a part in the nutritional failure of this protein.

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## THE DETERMINATION OF GLOBULINS IN BLOOD SERUM.

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The increasing importance attached to the proportion of globulins present in blood serum under varying conditions of health and nutrition as well as the importance of accurate knowledge as to the proportions present in antitoxic sera, has led recent investigators to inquire into the methods for their accurate determination. In the earlier methods for globulin determination<sup>1</sup> the globulins precipitated by the addition of an equal volume of saturated ammonium sulfate solution to the serum, or to a dilute solution of serum, were collected on a filter paper, washed with the salt solution, dried at 115°C., washed to remove salt, and then weighed. Obviously, this method is subject to many inaccuracies, chief of which is the difficulty in freeing the globulin precipitate of albumins and salt. Haslam<sup>2</sup> found that repeated precipitations at constant volume with equal volumes of saturated ammonium sulfate were required to free the globulins from albumins.

Berg<sup>3</sup> precipitated the globulins of blood serum by adding to the diluted serum an equal volume of saturated ammonium sulfate solution, and separated the precipitated globulins from the albumins by centrifuging. Only one separation was made. The precipitate was dissolved in water, coagulated by heat, filtered, washed, dried, and weighed.

In order to avoid the difficulties attending the washing and weighing of coagulated globulin precipitates, Reiss<sup>4</sup> and Robertson<sup>5</sup>

<sup>1</sup> Hammarsten, O., A text book of physiological chemistry, translated by Mandel, J. A., London, 1906.

<sup>2</sup> Haslam, H. C., *Biochem. J.*, 1912-13, vii, 492.

<sup>3</sup> Berg, W. N., *J. Agric. Research*, 1917, viii, 449.

<sup>4</sup> Reiss, E., *Beitr. chem. Physiol. u. Path.*, 1904, iv, 150.

<sup>5</sup> Robertson, F. B., *J. Biol. Chem.*, 1912, xi, 179.

devised a method for globulin estimation based upon the refractive index of the separated proteins. This method possesses numerous advantages, such as accuracy, simplicity, and rapidity, but has the disadvantage that an expensive and particularly sensitive refractometer is required, the lack of which has precluded, it is believed, its more general employment. More recently Cullen and Van Slyke,<sup>6</sup> with the same object in view, namely to avoid the washing and weighing of the globulin precipitates, have developed a method for the determination of globulins which is also applicable to the determination of other proteins. This method is based upon the determination, by the Kjeldahl method, of the protein nitrogen of the filtrate from the precipitation effected by the addition of an equal volume of a saturated ammonium sulfate solution to the serum solution; the ammonia of the ammonium sulfate present having been driven off previously by distillation with magnesium oxide from an alcohol-water solution.

Two methods for the determination of globulins, particularly in anti-hog-cholera serum have been developed in these laboratories. One has been used continuously since 1914, and the other for about 3 years. By either of these methods duplicate determinations checking within 0.1 per cent have been secured consistently, and prolonged use of the methods has indicated that accurate comparative results are obtainable with either method. The estimation of the accuracy and reliability of a method for the determination of globulins can be done most advantageously by a comparison of the results secured by the method in question with those obtained by other methods, it being understood, of course, that any method to merit consideration must be capable of yielding uniform results when applied to various lots of the identical serum. On the appearance of the description of Cullen and Van Slyke's method, tests were conducted that furnished evidence of the general reliability of the method. As the two methods, which have been used for some years in these laboratories, had always been found to yield accurate comparative results, the idea was suggested of comparing the relative merits of the Cullen and Van Slyke method and the methods used in these laboratories. It was for this purpose that this study was undertaken. The results showed that the two methods used in these laboratories,

<sup>6</sup> Cullen, G. E., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 587.

as well as the Cullen and Van Slyke method, all afford an accurate means of determining globulins. Inasmuch as the methods developed in these laboratories appear to give results in practical agreement with those obtained by the Cullen and Van Slyke method, and are, in some respects at least, less involved than the Cullen and Van Slyke method, it seems desirable, for the benefit of others interested, to describe them briefly, and to detail the comparative amounts of globulins found by the three different methods in identical lots of horse serum.

*Methods for the Determination of Globulins.*

*Method 1.*—König and Kisch<sup>7</sup> recommended, in 1889, the estimation of globulins by precipitating the globulins with saturated magnesium sulfate, and then determining the nitrogen in the precipitate by the Kjeldahl method. Accurate determinations by this method are impossible because of the difficulty experienced in attempting to wash albumins from globulin precipitates. On this account, an attempt was made to estimate the globulins by determining the nitrogen of the filtrate and by making the necessary calculations to determine the globulins by difference. The method is used as follows:

Two determinations are required: (1) Determination of total nitrogen. The total nitrogen is determined, using a 5 cc. portion of the serum, and is expressed in grams of nitrogen in 100 cc. of serum. (2) Determination of non-globulin nitrogen. The globulins are first removed from the solutions as follows: To 10 cc. of serum contained in a 100 cc. graduated flask, 60 to 70 cc. of a saturated magnesium sulfate solution are added and the contents thoroughly mixed by rotation—agitation—a producer of foam in protein solutions, is to be avoided. 12 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  are next added to complete the saturation, and the flask is allowed to stand, with intermittent rotation, until the crystals have dissolved, when the contents of the flask are diluted to the 100 cc. mark with a saturated magnesium sulfate solution.

After standing over night, the contents of the flask are filtered, a 20 cc. portion of the filtrate, which should be water-clear, is taken and the nitrogen in this determined by the Gunning method, using a reduced amount of  $\text{K}_2\text{SO}_4$ . The quantity of nitrogen found, of course, represents the quantity of nitrogen present as non-globulin nitrogen in 2 cc. of serum, and is calculated to grams per 100 cc. of serum.

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<sup>7</sup> König, J., and Kisch, W., *Z. anal. Chem.*, 1889, xxviii, 191.

*Calculation.*—The total nitrogen found less the non-globulin nitrogen, represents the globulin nitrogen. The factor 6.3 is used to convert the globulin nitrogen to globulins, and the results are expressed in grams of globulins per 100 cc. of serum.

*Method 2.*—This method has been used since 1914, in which year it was devised to determine the quantity of globulins present in anti-hog-cholera serum. While no originality is claimed, it is believed, that of the methods in which globulins are determined by direct weighing, the method as here described will yield more accurate results than are obtainable with any other similar method. The description follows:

To 10 cc. of serum contained in a 400 cc. beaker, 90 cc. of water are added, stirred, and then 100 cc. of a saturated ammonium sulfate<sup>8</sup> solution are added, with constant stirring. The beaker is covered with a watch-glass, allowed to stand until the globulins separate into thick masses, usually over night, and the contents are then filtered through 11 cm. hardened papers, the filtrate being returned to the paper if not clear. The solution either in the beaker, or funnel, is kept covered with watch-glasses.

After the filtration is complete the precipitate is dissolved, while the paper is in the funnel, with physiological salt solution. Solution is easily effected. Frequent additions of the salt solution are of course made, but the final volume should not exceed 100 cc. The solution of the globulins is measured in a cylinder, the volume brought to 100 cc., and an equal volume of a saturated ammonium sulfate solution added, using the same cylinder to measure the ammonium sulfate solution that was used to measure the globulin solution. The globulins are removed by filtration as before.

The globulins from the second precipitation are again dissolved in salt solution, the volume being brought to about 300 cc. The solution is brought to a boil, and 1 or 2 drops of a 10 per cent solution of acetic acid added if necessary to cause flocculation. The solution will foam tremendously if boiling is continued for any time, so as the boiling point is approached, a close watch to check foaming should be maintained.

After standing on a steam bath until the globulins have separated into a thick flocculent mass, the solution is filtered through a tared hardened (hardened papers must be used) paper, any protein remaining adhering to the walls of the beaker being completely removed. In order to remove retained sulfates, the precipitate is washed with hot water, about 200 cc. being used, and each portion being allowed to pass through the paper before the succeeding portion is added. The precipitate and paper are trans-

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<sup>8</sup> Only tested ammonium sulfate should be used. An ammonium sulfate, stated to be C. P., has been found which contained more than 1 per cent  $\text{NH}_4\text{HSO}_4$ . Needless to say, such an ammonium sulfate will ruin any protein determination.

ferred to a tared, aluminium weighing dish provided with a cover, and dried to constant weight, or until a loss of less than 1 mg. is shown after 3 hours drying, at 100° C. As the papers are very hygroscopic the dishes should be tightly covered when removed from the drying oven.

The Cullen and Van Slyke method<sup>6</sup> was used essentially as described in the Journal of Biological Chemistry. However, no determinations of non-protein nitrogen were made, the globulin nitrogen being calculated as the difference between the total nitrogen present less the non-globulin nitrogen. The various corrections were made. It may be mentioned that the globulins were separated by diluting 10 cc. of serum with 50 cc. of water, adding 50 cc. of a saturated ammonium sulfate solution and filtering. It was found also, that the bumping of the flask in the distillation of the free  $\text{NH}_3$  in the  $\text{MgO}$  distillation could be better controlled, if the  $\text{MgO}$ , water, and alcohol were added in this order, the flask being agitated after each addition, rather than if added in the order  $\text{MgO}$  followed by the required amount of 50 per cent alcohol-water.

Merck's  $\text{MgO}$  is specifically recommended by Cullen and Van Slyke, and was found by the writer to be superior to any other powdered magnesium oxide. However, with a commercial pharmaceutical preparation of milk of magnesia<sup>9</sup> the distillation proceeded more quietly than with Merck's and the amount of nitrogen in the reagents employed was lower than in the case of Merck's. In Cullen and Van Slyke's description of this method, the freedom of the distillate from ammonia in the  $\text{MgO}$  distillation is determined by testing the distillate with litmus paper. In the writer's experience, litmus papers have not proved reliable. Instead, the distillation is continued until 10 cc. of distillate do not neutralize 0.1 cc. of 0.1 N  $\text{H}_2\text{SO}_4$ , using methyl red as an indicator.

#### *Comparison of Methods.*

In order to compare the results obtained by these methods, the globulins present in three different lots of horse serum were determined by each of the three methods, all determinations being made in duplicate. The serum used was obtained by centrifuging defibrinated blood obtained from normal horses, and the first

<sup>9</sup> Phillips' brand was used.

precipitations were made in all cases while the serum was but a few hours old.

A determination of the ammonium sulfate remaining in the coagulated globulin precipitates obtained in Method 2, was made in most cases, but the amounts were too small to render corrections necessary.

The results are shown in Table I.

TABLE I.  
*Amounts of Globulins Determined by the Different Methods.*

Serum.	Method.	Grams of globulins per 100 cc. of serum.				General average.
		Duplicates.		Difference.	Average.	
		1	2			
A5	1	5.58	5.59	0.01	5.59	5.48
	2	5.55	5.48	0.07	5.52	
	Cullen and Van Slyke.	5.28	5.36	0.08	5.32	
A6	1	5.57	5.54	0.03	5.55	5.36
	2	5.55	5.40	0.15	5.48	
	Cullen and Van Slyke.	5.04	5.04	0.00	5.04	
A7	1	3.95	3.97	0.02	3.96	4.04
	2	3.98	4.02	0.04	4.00	
	Cullen and Van Slyke.	4.14	4.14	0.00	4.14	

The very close agreement in duplicates as determined by Method 1 and by the Cullen and Van Slyke method is noteworthy.

It may be remarked that no one method gave consistently high or low results, although the Cullen and Van Slyke method gave the lowest results in two of the three serums, and Method 1 gave the highest results in two serums. On the whole it may be said, that each method yields dependable results when judged either by comparing results of duplicate determinations by the same method, or by comparing results, on the same serum, obtained by the different methods.

#### *Effects of Dilution.*

It has been stated frequently that the amount of globulins that can be separated from a given serum, or serum solution, is dependent upon the dilution of the serum from which the globulins are

TABLE II.  
*Effects of Dilution.*

Serum.	Method.	Dilution.		Globulins per 100 cc.
		Serum.	Total volume at separation.*	
A5	1	cc.		gm.
		10	100	5.59
		5	100	5.18
	2	10	200	5.52
		5	200	5.22
		5	300	5.07
	Cullen and Van Slyke.	10	100	5.31
		5	100	5.40
	1	20	100	5.55
		10	100	5.29
		5	100	5.00
A6	2	10	200	5.48
		5	200	5.36
		5	300	5.43
	Cullen and Van Slyke.	20	100	5.15
		10	100	5.14
		5	100	4.75
	1	20	100	3.64
		10	100	3.96
		5	100	4.03
	2	20	200	4.20
		10	200	4.00
		5	200	3.99
A7	Cullen and Van Slyke.	20	100	4.03
		10	100	4.14
		5	100	3.94

\* Precipitations effected by saturated solutions of magnesium sulfate or half saturated solutions of ammonium sulfate, depending upon the method used.



precipitated, but, usually, detailed data supporting this statement have been lacking. In order to determine if the dilution at which globulins are precipitated has any influence on the amounts of globulins that will be obtained, globulins were precipitated from serum solutions diluted not only in accordance with the description of the methods previously given, but also in dilutions greater and less than those recommended. Thus, in Method 2, dilutions of 10 cc. of serum in 100 cc. of saturated magnesium sulfate solution are specified; separations were made not only at this dilution but in dilutions of 5 cc. of serum in 100 cc. of sulfate solution, and 20 cc. of serum in the same amount of sulfate solution. Table II shows the serums employed, the dilutions at which the separations were made, the methods used, and the results obtained. All determinations were made in duplicate, and with four exceptions all duplicates checked within 0.1 gm. of globulins per 100 cc.

It is apparent that, on the whole, there are only slight variations, within the dilutions employed, in the amounts of globulins found, indicating that the amounts of globulins separated from albumins are but slightly, if at all, dependent upon the concentration of protein or upon similar factors. In other words, it is indicated that globulins are a rather distinct entity. However, a slight tendency for the amounts of globulins separated to vary with the dilution at which separation is effected, is recognized, and in recognition of this complication, separations of globulins should be effected always at definite unvarying dilutions.

#### *Relative Advantages of Methods.*

From general experience with these methods, the following conclusions regarding their comparative advantages appear to be justified:

1. Duplicate determinations agree slightly more closely when made by the Cullen and Van Slyke method, than when made by the other two methods, and the results obtained by the Cullen and Van Slyke method appear perhaps to be less influenced by dilution. The Cullen and Van Slyke method is more complicated and requires more care than the other methods. Accurate determinations cannot be made by this method unless bumping during distillation of the free ammonia is prevented.

2. Method 1, the method in which magnesium sulfate is used, is the simplest of the three, and requires less attention and less time than the others. Experience indicates that the results obtained by this method will be dependable within the limits set forth in the tables.

3. Method 2 requires more time and does not yield quite as accurate results as the other methods. This method has been found useful when a large number of determinations are to be made.

4. Considering the nature of the substance for the estimation of which these methods are used, the results obtained by the different methods are surprisingly concordant, and it is believed that any one of the three methods may be recommended for the accurate determination of globulins, provided that it is used in the manner indicated.

#### SUMMARY.

1. Two previously undescribed methods for the estimation of globulins are given.

2. Results secured by these methods in comparison with each other and with the results obtained by the recently published method of Cullen and Van Slyke are shown.

3. A study was also made of the effects of varying the dilution at which globulins are precipitated.



# ON THE DETERMINATION OF THE TOTAL OXYGEN-COMBINING POWER OF THE BLOOD IN THE VAN SLYKE APPARATUS.

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In the original paper Van Slyke gives the following procedure for the determination of the total oxygen-combining power of the blood (hemoglobin):<sup>1</sup> 3 or more cc. of blood are introduced into a separatory funnel or bottle and distributed in a thin layer about the inner wall so that maximum contact with the air and complete saturation of the hemoglobin with oxygen are assured. The vessel is rotated for a few minutes so that the blood is kept in a thin layer, or it may be shaken for 15 minutes or longer with a mechanical shaker. The saturated blood is transferred to a heavy test-tube or cylinder, from which a sample is drawn into a pipette and run into the apparatus.

We have modified this in a way which, we think, affords a more convenient procedure. 6 cc. of water containing 2 to 3 drops of octyl alcohol and 0.3 cc. of 1 per cent saponin<sup>2</sup> are run into the apparatus. After the usual evacuation this is drawn down and trapped in the wide branch of the apparatus below the lower stop-cock. The stop-cock is turned and mercury is run very slowly upwards through the apparatus in order to collect the film of water left on the inside. The water is then run out of the left side of the upper part. The upper stop-cock is now turned and mercury is run into the bottom of the cup. If any moisture is left in the cup from the introduction of water it is dried by filter paper. 2 cc. of blood are now introduced into the cup and drawn down almost to the bottom of the 50 cc. chamber. The apparatus

<sup>1</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.

<sup>2</sup> Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, 1921, xlix, 1.

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is now shaken for  $\frac{1}{2}$  minute by hand or for from 2 to 3 minutes by a mechanical shaker. The upper stop-cock remains open. Thereby the blood is saturated. Mercury is again run up into the 50 cc. chamber collecting the blood at the top. When the blood column reaches the upper stop-cock, this is closed. The stop-cock is now turned so that the previously trapped air-free water is allowed to rise into the chamber. The lower stop-cock is closed and the apparatus turned upside down once in order to mix the water and blood. After  $\frac{1}{2}$  minute the blood is laked. Saturated potassium ferricyanide is now added and the determination made as described by Van Slyke in 1918 and modified by Van Slyke and Stadie in 1921.

The advantage of our procedure is that it requires less blood and can be done in a shorter time than the original method. There is, furthermore, no possibility of introducing any error by evaporation during the saturation of the blood in the separatory funnel.

# THE NUTRITIONAL REQUIREMENTS OF BABY CHICKS.

## II. FURTHER STUDY OF LEG WEAKNESS IN CHICKENS.\*

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(Received for publication, April 14, 1922.)

In 1920 we published (1) data recording our experience in the rearing of baby chicks on "synthetic" rations, and the relation of the factor of roughage in the diet of this species to so called "leg weakness." Our data confirmed the earlier observations of Osborne and Mendel (2) to the effect that under conditions of confinement a roughage factor—preferably paper among those tried—would greatly aid in avoiding the complicated syndrome known to poultrymen as leg weakness. Yet in all our experience in the use of the chicken for laboratory purposes there were failures in growth not only with charcoal, dirt, agar, and fullers' earth as a roughage, but also some failures with paper, even when all other factors of nutrition appeared to be adequately supplied.

We have studied the problem further and our results indicate that a factor of primary importance in avoiding trouble in the rearing of baby chicks under confinement is provision of an ample supply of the fat-soluble vitamine or more accurately those vitamins contained in cod liver oil. Our work has been with cod liver oil and we are unable to say whether our results are to be interpreted as due to the influence of the fat-soluble vitamine alone or to this vitamine plus the hypothetical antirachitic vitamine. The rate of growth attainable by this species is considerably greater than that of the rat and very probably its demand for the water-soluble vitamine is also large, but it appears that it is not greater than can be provided by a cereal grain such as white

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

corn and an *ad libitum* supply of skimmed milk. With these materials as the sole source of water-soluble and antiscorbutic vitamins, we have had excellent and uniform rates of growth of baby chicks of an initial 40 gm. weight to 700 gm. weight, provided the vitamine complex of cod liver oil (the fat-soluble vitamine?) was also available in comparatively generous quantities.

No leg weakness developed in these cases. The birds were unusually active and vigorous and were pronounced normal specimens by experienced poultrymen. When cod liver oil was withheld and the sole source of all vitamins was restricted to white corn and skimmed milk from the time of hatching, growth, with few exceptions, ceased in 4 to 6 weeks, followed by sudden death. Occasionally a bird confined to this ration would grow for a longer time, exhibiting the ruffled feathers, squatting position, edema of the eyes, and external symptoms defined as leg weakness, but ending ultimately in premature death.

We have been so impressed with these recent data that we are inclined to explain the considerable variations we have had in rearing baby chicks on synthetic as well as natural diets to a variation in the vitamine content of the butters used in making the synthetic rations and to the comparatively low or unavailable supply of this factor in the natural food materials used. We do not abandon the idea of a roughage as a necessary ballast and aid in the rearing of this species; but our available data do show that with white corn, skimmed milk *ad libitum*, common salt, calcium carbonate, a litter of shavings, and confinement it is possible to rear baby chicks to maturity with no indication of physical weakness provided cod liver oil is added to the diet. Evidently the demand by the chick for the fat-soluble vitamine is comparatively larger than that made by the rat during the early periods of growth. With 15 to 20 per cent of butter fat in the rations as the only source of the fat-soluble vitamine and all other nutritional factors provided, we have seen baby chicks fail to make normal growth.

Further, where chicks were started, as soon as hatched, on a mixed and complex dietary and then at 6 weeks of age and approximately 200 gm. of weight transferred to a low fat-soluble vitamine ration, such as white corn and skimmed milk, the progress of growth continued rapidly for 3 to 5 weeks, but gradually

slowed down, with the development of symptoms not unlike those described as leg weakness. In other words, rapid growth on the above ration was possible for a time with older birds through the use of the stored vitamine and the small amount of this factor available in the food, thereby prolonging life with an opportunity for the development of the syndrome of leg weakness into a lingering and distinctly chronic state.

#### EXPERIMENTAL.

We do not think it necessary to record the many trials, with some successes and some failures, that we have made in attempts to rear baby chicks under confinement, particularly at normal rates and with uniform results. This statement applies particularly to our work with synthetic rations, but also in a measure to work with natural foodstuffs. It is especially in the direction of uniformity of results that our work has been most checkered. One or two individuals in a group might grow well, while the remaining number would fail. No real success in the use of this species as a laboratory animal can follow until it is possible to rear normally a high percentage of individuals from any one hatch.

In the experiments recorded in this paper a hatch of 57 pure-bred Rhode Island Red chicks was divided into three groups of 19 each. The birds were confined indoors and to limited runs with shavings as a litter.

Group 1 received as a mash a mixture of 97 parts of white corn, 2 parts of calcium carbonate, 1 part of sodium chloride; skimmed milk was allowed *ad libitum*.

Group 2 received the same ration as Group 1, but to which were added 50 gm. of cod liver oil per kilo of grain-salt mixture. The cod liver oil was mixed intimately with the ration. We have tried feeding this material by pipette, but this led to loss of appetite and ultimate starvation. Where the cod liver oil was mixed with the ration and the newly hatched chick placed on this ration as soon as it was ready to take food, we had no trouble whatever with consumption.

Group 3 only served as a check and received a great complexity of diet. This consisted of a mash of 33 parts of bran, 32 parts of yellow corn, 32 parts of middlings, 1 part of charcoal, and 2 parts



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of fish scraps; whole milk was allowed *ad libitum*; a scratch mixture of 50 parts of yellow corn, 25 parts of wheat, and 25 parts of oats was also allowed. This group was useful to us only as serving as a source of partly grown individuals to be used in later phases of our experimental plan.

TABLE I.

*Record of Weights of Group 1, Fed White Corn, Skimmed Milk, CaCO<sub>3</sub>, and NaCl.*

No.	Initial weight.	2 weeks.	4 weeks.	6 weeks.	8 weeks.	Remarks.
	gm.	gm.	gm.	gm.	gm.	
1	34	80	125	150, dead.		
2	33	80	140	120 "		
3	32	75	125	110 "		
4	34	70	90, dead.			
5	37	85	130 "			
6	40	85	140 "			
7	34	85	140	160, dead.		
8	32	75	140	190 "		
9	37	85	160	270	360, dead.	
10	32	75	105			Taken for blood test.
11	35	70	95			" " " "
12	35	65, dead.				
13	36	75	100, dead.			
14	35	75	120	110, dead.		
15	34	85	135, dead.			
16	35	95	115			Taken for blood test.
17	32	80	120			" " " "
18	34	85	120			" " " "
19	39	85	125, dead.			

In addition to the calcium carbonate included in the rations of Groups 1 and 2, limestone grits were allowed all the birds. No water was given—this being derived wholly from the skimmed milk.

In Tables I and II are recorded the growth records of Groups 1 and 2. It will be noted that with one exception all of the animals of Group 1 died within 6 weeks after the initiation of the experiment. Some died suddenly; others lingered, showing ruffled feathers, a tendency to squat, and symptoms of leg weakness.

Group 2, receiving the cod liver oil in addition to the basal ration, made with two exceptions uniform and rapid growth. There was no indication in this group of any physical difficulties whatever. The uniformity of results impressed us as indicating the effectiveness for this species of a bountiful supply in the diet of

TABLE II.

*Record of Weights of Group 2, Fed White Corn, Skimmed Milk, CaCO<sub>3</sub>, NaCl, and Cod Liver Oil.*

No.	Initial weight.	2 weeks.	4 weeks.	6 weeks.	8 weeks.	10 weeks.	11 weeks.	Remarks.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
20	37	90	170	310	460	640	790	
21	35	65	135	250	400	580	680	
22	37	80	160	270	390	540	660	
23	35	80	145	220	310	430		Taken for blood test.
24	34	100	170	290	440	690	870	
25	31	50	100					Taken for blood test.
26	34	75	145					" " " "
27	34	70	90					" " " "
28	34	80	160	260	390	580	680	
29	35	70	135	210	320	410	450	
30	34	80, dead.						
31	37	85	155	265	380	520	630	
32	34	65	90, dead.					
33	32	85	155	280	510	640		Taken for blood test.
34	32	80	120					" " " "
35	35	85	175	320	500	750	870	
36	32	80	155	265	400	540	670	
37	33	70	120					Taken for blood test.
38	39	80	155	220	380	520		" " " "

the vitamins of cod liver oil. We made no examination of the skeletons to determine whether they were normal or not. We do not claim that the ration used was an ideal one in all respects and that the optimum amounts of calcium and phosphorus were provided.

At 4 weeks of age, 5 individuals from Group 1 as well as 5 individuals from Group 2 were taken for blood analysis. We desired to know whether there was the same correlation with re-

spect to the inorganic phosphorus content of the blood in these groups of animals as had been observed by Howland and Kramer (3) in children suffering from rickets.

The method used for the determination of inorganic phosphorus was that described by Marriott and Haessler (4). Food was withheld from these birds 12 hours before bleeding to insure that the blood would not be contaminated with food from the crop as it flowed from the severed necks into test-tubes. In Table III the results of these analyses are given.

The data on blood analysis indicate that, in respect to forms of phosphorus, changes were occurring in this species similar to those observed in rachitic children. The record of Bird 16 is an excep-

TABLE III.  
*Inorganic Phosphorus per 100 Cc. of Serum of Groups 1 and 2.*

No cod liver oil, Group 1.				Cod liver oil, Group 2.			
No.	Age.	Weight.	Phosphorus.	No.	Age.	Weight.	Phosphorus.
	<i>wks.</i>	<i>gm.</i>	<i>mg.</i>		<i>wks.</i>	<i>gm.</i>	<i>mg.</i>
10	4	105	1.93	25	4	100	2.50
11	4	95	1.07	26	4	145	5.15
16	4	115	2.80	27	4	90	2.73
17	4	120	1.40	34	4	120	3.80
18	4	120	1.73	37	4	120	3.80

tion, not showing a lower inorganic phosphorus content of its blood than shown by two individuals receiving the cod liver oil. We do not want to imply that these animals were suffering from rickets and that leg weakness in poultry is rickets until further data are accumulated. But it is significant that cod liver oil, a specific for rickets, completely prevented, in a very considerable group of individuals, the complex known to poultrymen as leg weakness.

The six birds from Group 3 which had received a complex diet and were transferred at weights of 200 gm. or more to the low fat-soluble vitamine ration given Group 1, grew well for 3 to 5 weeks after the transfer, but after that time began to fail. The feathers became ruffled, and in some individuals the crop became compacted, squatting was frequent, and many of the gross symptoms of leg

weakness presented themselves. After these chickens had been on the low fat-soluble vitamine ration for 4 weeks and noticeable signs of failure were apparent, three individuals, Nos. 39, 40, and 41, were taken for the determination of inorganic phosphorus in the blood. These three birds had begun to lose weight; the other three had not. Nos. 42, 43, and 44—those not losing weight at 4 weeks after the transfer—were held on the ration for a longer time, or until they began to fail when they were sacrificed for

TABLE IV.

*Record of Weights and Inorganic P in Serum of Individuals Transferred from Complex Diet to Low Fat-Soluble Vitamine Diet at Weights of 200 Gm. or More (Group 3). Also Record of Three Individuals from Group 2 Receiving Cod Liver Oil and 10 Weeks Old.*

No.	Trans-fer weight.	2 weeks later.	4 weeks later.	6 weeks later.	Group 2, 10 weeks old.	Inor-ganic P per 100 cc. of serum.	Remarks.
	gm.	gm.	gm.	gm.	gm.	mg.	
39	300	470	390			2.41	Killed for blood.
40	230	410	400			1.59	" " "
41	250	460	450			2.41	" " "
42	245	390	485	470		3.75	" " "
43	220	330	440	430		3.70	" " "
44	220	340	445	435		2.80	" " "
23					430	7.13	" " "
33					640	5.82	" " "
38					520	9.40	" " "

a blood analysis. At the same time three individuals from Group 2, receiving the cod liver oil, were killed for blood analysis to serve as comparisons. The records of the weights of the entire group of transferred birds, as well as the data on blood analysis of this group and the blood analysis of those from Group 2, killed at the same time that Nos. 42, 43, and 44 were killed, are given in Table IV.

The amount of inorganic phosphorus in the blood of this transferred group tends to run low and the data harmonize fairly well with the observations on the group started at hatching and receiving no cod liver oil (Table III). There are some variations and we are not sure whether these are to be explained as inherent faults in the method of determining inorganic phosphorus or to

the shorter time of exposure of the individuals in this group to the diet free from cod liver oil. The blood of the birds receiving the cod liver oil was distinctly and uniformly higher in inorganic phosphorus than the blood from the birds not receiving cod liver oil. These additional data support the view that in this species, as has been reported for infants, the amount of inorganic phosphorus in the blood may be below the normal amount where the supply of the fat-soluble vitamine (?) or the supply of particular mineral constituents of the diet is low in amount.

#### SUMMARY.

1. We have been able to rear baby chicks under confinement to weights of 800 gm. or more where the diet consisted of white corn, skimmed milk, a simple salt mixture, and cod liver oil. A litter of shavings was provided. Vigorous and apparently normal birds were produced. We did not carry this group longer than 11 weeks which usually is past the critical stages of early growth.

2. It appears that this species requires a liberal supply of the vitamins of cod liver oil during its most active period of growth, but that the water-soluble and antiscorbutic vitamine requirement can be met by the amounts contained in a cereal grain and skimmed milk. However, it is possible that under certain conditions the water-soluble vitamine supplied by a cereal grain would be too low in amounts. This point is under investigation.

3. When the cod liver oil was omitted from the ration the animals died in 4 to 6 weeks. The gross symptoms in some cases were those described by poultrymen as leg weakness. In other cases death was sudden. The inorganic phosphorus content of the blood of the birds in this group was low as compared with those receiving cod liver oil. This condition has been observed in infants suffering from rickets.

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## A METHOD FOR THE DIRECT DETERMINATION OF URIC ACID IN URINE.

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In a previous paper one of us<sup>1</sup> described a method for the direct determination of uric acid in tungstic acid blood filtrates, based upon the use of an arsenic phosphoric tungstic acid reagent. On account of the high specificity of this reagent for uric acid we were led to try its application for uric acid determination in urine without previous separation of the uric acid. The results obtained in this connection have proved very satisfactory. With the new procedure the determination of uric acid in urine is quite as simple as the creatinine determination.

The new procedure is as follows.

### *Solutions Required.*

1. *Reagent.*—The reagent employed is the one used in the new procedure for the direct determination of uric acid in blood,<sup>1</sup> and is prepared as follows. 100 gm. of pure sodium tungstate (Primos brand, Merek's, or J. T. Baker's c. p. product) are placed in a liter Pyrex flask and dissolved in about 600 cc. of water. 50 gm. of pure arsenic acid ( $\text{As}_2\text{O}_5$ ) are now added, followed by 25 cc. of 85 per cent phosphoric acid and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for about 20 minutes, cooled, and diluted to 1 liter. The reagent appears to keep indefinitely.

2. *Sodium Cyanide.*—A 5 per cent solution of sodium cyanide, which should be prepared fresh once in about 2 months, is used.

3. *Uric Acid.*—A standard solution of uric acid, acidified with hydrochloric acid, containing 0.2 mg. of uric acid in 10 cc., is

<sup>1</sup> Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.

employed. This solution may be readily prepared by dilution of the phosphate standard uric acid solution described by Benedict and Hitchcock,<sup>2</sup> as follows. 50 cc. of the phosphate standard solution (containing 10 mg. of uric acid) are measured into a 500 cc. volumetric flask and diluted to about 400 cc. with distilled water. 25 cc. of dilute hydrochloric acid (made by diluting 1 volume of the concentrated acid with 9 volumes of water) are added, and the solution is diluted to 500 cc. and mixed. This dilute standard solution should be prepared fresh from the phosphate standard every 10 days to 2 weeks.

*Procedure.*—The urine is so diluted that 10 cc. will contain between 0.15 and 0.30 mg. of uric acid. (Usually a dilution of 1 to 20 is satisfactory.) 10 cc. of the diluted urine are measured into a 50 cc. volumetric flask, 5 cc. of the 5 per cent sodium cyanide solution are added from a burette, followed by 1 cc. of the arsenophosphotungstic acid reagent. The contents of the flask are mixed by gentle shaking, and at the end of 5 minutes diluted to the 50 cc. mark with distilled water and mixed. This blue solution is then compared in a colorimeter with a simultaneously prepared solution obtained by treating 10 cc. of the standard uric acid solution (0.2 mg. of uric acid) in a 50 cc. flask with 5 cc. of the sodium cyanide solution, 1 cc. of the reagent, and diluting to the mark at the end of 5 minutes. The calculation of results is very simple. The reading of the standard (15 or 20 mm.) divided by the reading of the unknown, and the result multiplied by 0.2, gives the milligrams of uric acid contained in the 10 cc. of diluted urine used in the unknown.

Some explanatory remarks in connection with the technique may be of value. The proportional depth of color for uric acid

<sup>2</sup> The phosphate standard solution of Benedict and Hitchcock is prepared as follows. Dissolve 9 gm. of pure crystallized disodium hydrogen phosphate together with 1 gm. of monosodium dihydrogen phosphate in 200 to 300 cc. of hot water. Filter if not perfectly clear and make up to about 500 cc. with hot water. Pour this warm solution upon exactly 200 mg. of uric acid suspended in a few cc. of distilled water in a liter flask and shake gently until all the uric acid passes into solution. Cool the solution, add exactly 1.4 cc. of glacial acetic acid, dilute to 1 liter, and mix. 5 cc. of chloroform are then added to prevent bacterial growth. 5 cc. of this standard solution contain 1 mg. of uric acid. Unless kept in an excessively warm place the solution may be relied upon to keep for 2 months.

concentrations varying between 0.15 and 0.30 mg. is almost absolutely exact under the conditions indicated, when 0.2 mg. of uric acid is used as the standard. Outside of this range the results are not quite satisfactory, and cannot be materially improved by greater or less dilution of the unknown. It is essential that the volume of the unknown and of the standard be the same during the period of the reaction. This volume may be 15 cc., instead of the 10 cc. indicated, if desired for any special reason, but in such cases the standard must be diluted to the same volume before addition of the reagents.

It may be noted that the procedure for urine differs from that proposed for blood in that the reacting mixture is not heated in the case of the urine determination. This is because in this latter determination we have a relatively large quantity of uric acid present, and perfectly satisfactory results can be obtained by making use of the equilibrium point of the reaction reached in 5 minutes under the fixed conditions. If it were necessary, owing to lack of sufficient urine to supply 0.2 mg. of uric acid, we could undoubtedly work with about one-tenth of this amount under conditions similar to those adopted for the blood.

About 50 samples of human urine have been analyzed by both the new method and the Benedict-Hitchcock<sup>3</sup> procedure. Many of the samples examined contained large quantities of albumin, glucose, or diacetic acid. None of these substances interfere with the new method to the slightest degree except albumin. If this latter substance is present in appreciable amounts it is best to remove it by heat coagulation in presence of a drop of acetic acid, and filtration, prior to making the determination. In no urine analysis which we have made have we encountered turbidity, or the slightest difficulty in matching the shade of color obtained in the standard and the unknown. Comparative figures obtained on 25 samples of human urine (selected at random from all those we have analyzed) by the new method and by the Benedict-Hitchcock procedure are given in Table I. We also analyzed many of these samples by the new procedure of Folin and Wu,<sup>4</sup> and obtained figures essentially duplicating those obtained by the two methods reported. Since the Folin-Wu method appears to us

<sup>3</sup> Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.

<sup>4</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 459.



to offer no advantage in technique over the Benedict-Hitchcock procedure, and relatively weak colors are obtained for the final

TABLE I.

*Showing Comparative Figures for Uric Acid in Human Urine by the Proposed Method and by the Benedict-Hitchcock Modification of the Folin-Denis Method.*

Sample No.	Uric acid.		Remarks.
	Benedict-Hitchcock procedure.*	Proposed method.*	
	mg.	mg.	
1	586	569	Normal.
2	293	285	"
3	829	855	Albuminuria.
4	881	851	Diabetes.
5	745	751	"
6	416	392	Albuminuria.
7	680	660	Indicanuria.
8	653	639	"
9	684	680	Normal.
10	837	813	"
11	416	401	"
12	1,010	988	"
13	821	831	"
14	421	414	Albuminuria.
15	660	650	Normal.
16	590	575	Diabetes.
17	296	270	"
18	580	550	Normal.
19	832	860	"
20	190	180	"
21	490	482	Diabetes.
22	530	524	Normal.
23	870	876	"
24	290	282	Albuminuria.
25	665	653	Normal.

\* The figures represent milligrams of uric acid per liter of urine.

reading we have not included the figures by this method in the table.

An inspection of the figures reported in Table I shows, we believe, very satisfactory agreement between the results by the two processes. That there is no substance, which reacts in the new

method, other than uric acid present in the urine, is clearly shown by the definite tendency to lower slightly the figures shown by the new method. It is also interesting to note in this connection that dogs' urine, which is relatively rich in phenol bodies, but very low in uric acid content (except the urine of the Dalmatian dog) gives only a trace of color by the new method, corresponding to an output of only a very few milligrams of uric acid for 24 hours. Thus the new method appears suitable for work with urines of widely varying species, though we have not yet investigated this point in any detail.

The fact that urines, in spite of their high content of phenol-reacting bodies, contain no substance which appears to interfere with the direct use of the new reagent to the slightest degree, adds very definite support to the view that this reagent is safe for the direct determination of uric acid in blood filtrates.

We are at present studying the possible applications of arsenic molybdic tungstic compounds to the determination of phenols in blood and in urine.



## THE THRESHOLD OF KETOGENESIS.\*

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(Received for publication, April 11, 1922.)

It is recognized that diets so unbalanced as to contain greatly disproportionate amounts of fat lead to the formation and excretion of acetoacetic acid,  $\beta$ -hydroxybutyric acid, and acetone. The accumulation of these substances depends on the ratio existing between the ketogenic substances, particularly fatty acids and the glucose of the metabolizing foods. That ratio at which significant ketosis first appears may be called the threshold of ketogenesis. Accurate knowledge of the value of this threshold is a matter of importance in gauging the safety of diets high in fat such as may be used in diabetes. Zeller (19), Lusk (4), Ringer (7), Woodyatt (17, 18), Shaffer (8, 9), Palmer (6), Hubbard (2), Hubbard and Wright (3), and others have contributed data to the subject. Woodyatt (18) has discussed the problem with great lucidity and Shaffer (8, 9) has reported investigations which have stimulated thought and study.

With mixtures of varying proportions of acetoacetic acid and glucose in alkaline hydrogen peroxide, Shaffer (8, 9) beheld in the test-tube what he considered to be an *in vitro* analogy to the action of glucose in abolishing or preventing the formation of acetoacetic acid in man. When the proportion of acetoacetic acid to glucose in such mixtures was that of 1 (or possibly 2) molecules of acetoacetic acid to 1 of glucose, the former substance was completely oxidized. When the proportion of glucose was less, a considerable fraction of acetoacetic acid escaped oxidation. In these papers, Shaffer calculated the number of molecules of ketogenic material and the number of molecules of glucose in the determined metabolism of several human subjects and concluded

\* Presented before the American Society for Clinical Investigation at Washington, D. C., May, 1922.

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that the maximal ratio compatible with the oxidation of the ketogenic compounds was reached when the number of ketogenic molecules just equalled the number of glucose molecules, a ratio of 1:1. He subsequently modified this conclusion (11), and now considers that each glucose molecule is ketolytic for 2 molecules of acetoacetic acid, a 2:1 ratio. Woodyatt (18) and likewise Hubbard and Wright (3) have based working formulas for calculating diets on a 1:1 ratio.

Our interest in the ketogenic threshold was stimulated by Shaffer's first paper and we were encouraged to pursue the study because at the time we were trying, for diabetic patients, diets relatively high in fat such as had been proposed by Newburgh and Marsh (5). We found that we were feeding mixtures of food in which the ratios of ketogenic molecules to glucose molecules, as calculated by Shaffer's method, were considerably higher than 1:1. Despite this we were not provoking any appreciable ketosis. This was true at least under the conditions observed. The protein in the diets did not exceed 1 gm. for each kilo of the subject's body weight and the total calories of the diets were not above the caloric needs of the patients. We recognized, however, that these food mixtures did not necessarily represent the composition of the mixtures which were actually burning and the next step was an endeavor to determine the value of the ratio between the fatty acid molecules and the glucose molecules in the burning mixture. Our first thought was that the non-protein respiratory quotient and the Zunz-Schumburg (4) tables would give us this information, but in this we were disappointed. We depended, for our respiratory metabolic data, on methods involving indirect calorimetry and short period observations; respiratory quotients obtained under these circumstances cannot mirror the relative proportions of fat and carbohydrate engaged in the total metabolism of 24 hours. This has been discussed in another communication (16) and is in agreement with conclusions reached by Shaffer (10). A more reliable method of determining the composition of the burning mixture is the following:

The basal metabolic calories may be accurately measured by short period observations, and if the patient is confined to bed, the extra energy exchange due to the specific dynamic action of food and that resulting from movements in bed may be estimated as 20 per cent of the basal calories with a reasonable degree

of correctness. An approximation of the total metabolism for 24 hour periods may thus be reached. The nitrogen elimination for 24 hour periods being also determinable, the calories from the protein metabolism are known, each gram of urinary nitrogen representing 26.51 calories. If a low carbohydrate quota (60 gm. or less each day) is maintained for several days, it is reasonably safe to assume that all of the carbohydrate included in the diet is burned. The calories from the fat metabolism are obtained by subtracting the sum of the calories of the protein and the carbohydrate metabolism from the total calories of the day.

Employing the values of fat, protein, and carbohydrate secured in this manner, we calculated the ketogenic ratios of a group of patients on high fat diets. The results in one such patient have been reported elsewhere (15, 16). This patient was a young woman with diabetes of the acute, progressive type, and on two occasions the dextrose-nitrogen ratio of the urine reached 3.65:1 and continued at this height for 4 consecutive days. On both occasions, acetone bodies accumulated in the blood and coma threatened. The rising acidosis was checked by a diet in which the proportion of fat was very high, and an analysis of the mixture actually metabolized after acidosis was controlled revealed ratios of ketogenic molecules to glucose molecules of 1.5:1 and 1.8:1, respectively.

The present report is concerned with the determined composition of metabolizing food mixtures in sixteen other patients. Three of these patients had epilepsy but were, in other respects, apparently normal. Thirteen had diabetes; two of these suffered from mild, acute infections. None of the epileptic patients had convulsions and the diabetic patients were without glycosuria at the time of the investigation. The patients were confined to their rooms during the 3 days preceding the test; on the day before the test and on the day of the test, they remained in bed. During this period, each received a constant diet. The food consisted of rice, soy bean bread enriched with fat, butter, and cream; its composition was calculated from the Atwater-Bryant tables. The daily food allowance did not exceed in caloric value the energy needs of the patient and the protein quota was less than 1 gm. for each kilo of body weight. Precautions were taken to secure accurate collections of urine. Daily determinations of urinary nitrogen were made by means of the Kjeldahl method.

Van Slyke's methods were followed for acetone bodies of the urine and blood (Van Slyke and Fitz, 14), and for the carbon dioxide-combining power of the plasma. The basal respiratory metabolism and respiratory quotients were secured by the gasometer method as described by Boothby and Sandiford. The calculations of metabolizing mixtures and the ratios of ketogenic molecules to glucose molecules in these mixtures are based on assumptions, which, for the sake of clarity will be restated.

*Assumptions Involved in Calculating the Metabolizing Mixture.*

1. The total energy exchange, or total metabolism, is accurately represented by the basal calories for 24 hours plus 10 per cent for the specific dynamic action of food and 10 per cent for movements. This assumption is justified if the patient is confined to bed during the test day and for 1 or more preceding days, as was the case in these experiments.

2. All glucose derived from the food (carbohydrate, protein, and fat) is burned. This assumption is justified, provided the subject has received relatively little carbohydrate and protein for several days preceding the test, and provided the diet does not exceed the energy and nitrogen requirements of the subject, as was the case in these experiments. The total amount of carbohydrate in the diet of a patient with diabetes must not exceed the tolerance for carbohydrate if this assumption is to hold. Furthermore, carbohydrate starvation may very well increase the avidity with which glucose is stored as glycogen, so that less than the amount of glucose derivable from the food will actually burn. Errors of this kind would tend to give erroneously low values for calculated ketogenic ratios.

3. No carbohydrate from endogenous sources (glycogen) is burned. Such an assumption is naturally precarious. It is presumed, however, that the glycogen stores of patients who are on a régime very low in carbohydrate are retained, tenaciously. An error from this source would tend to give erroneously high values for calculated ketogenic ratios.

4. The nitrogen in grams in a 24 hour specimen of urine multiplied by 26.51 is the number of calories from the protein metabolized.

Given: the total metabolism  $M$ , the grams of carbohydrate of the food  $C$ , and the grams of nitrogen of the urine  $N$ . The amount

of fat  $F$  in the metabolizing mixture is calculated from the equation:

$$F = \frac{M - (C \times 4.1) + (N \times 26.51)}{9.3}$$

The values for carbohydrate and fat thus obtained are used in the calculation of the ratio between the ketogenic molecules and the glucose molecules as follows:

*Assumptions Involved in Calculating the Ratio of Ketogenic Molecules to Glucose Molecules, (the K : G ratio)<sup>1</sup> (Shaffer 8, 9).*

1. 1 gm. of fat, molecular weight 874 =  $\frac{1}{874} \times 3 = 0.00343$  gm. molecules of ketogenic fatty acid. Let  $a$  = grams of fat  $\times 0.00343$ .

2. 1 gm. of urinary nitrogen = 0.01 gm. molecules of ketogenic substance. Let  $b$  = grams of nitrogen of 24 hour urine  $\times 0.01$ .

This is based on Osborne's analysis of ox muscle as modified by Lusk (4) and is derived as follows:

*Ketogenic Amino-Acids in 100 Gm. of Muscle Protein.*

	Grams.	Molecular weight.	Gm. molecules.
Leucine.....	14.3	131	0.109
Phenylalanine.....	4.5	165	0.027
Tyrosine.....	4.4	181	0.024
Sum for 16 gm. of nitrogen.....			0.160
Sum for 1 gm. of nitrogen.....			0.010

3. 1 gm. of glucose (carbohydrate), molecular weight 180 =  $\frac{1}{180}$  or 0.00556 gm. molecules of glucose. Let  $c$  = grams of carbohydrate  $\times 0.00556$ .

4. 1 gm. of nitrogen of the urine =  $\frac{1}{180} \times 3.65$  or 0.02 gm. molecules of glucose. This is based on the assumption that 3.65 gm. of glucose are liberated from protein with each gram of nitrogen. Let  $d$  = grams of nitrogen of 24 hour urine  $\times 0.02$ .

5. 1 gm. of fat, molecular weight 874 =  $\frac{1}{874}$  or 0.00114 gm. molecules of glycerol  $\div 2 = 0.00057$  gm. molecules of glucose. Let  $e$  = grams of fat  $\times 0.00057$ .

$$\text{The K:G ratio} = \frac{a + b}{c + d + e}$$

<sup>1</sup> Recently Shaffer (11) has revised some of the assumptions involved in these calculations, but inasmuch as his original assumptions are in closer agreement with those generally accepted, we have preferred to use them at this time. The results of the calculations do not differ materially from what they would be if his revised assumptions were adopted.



TABLE I—Ratio of Ketogenic Molecules to Glucose

Case No.	Date.	Age.	Sex.	Height.	Weight, naked.	Diagnosis.*	Food, 3 preceding days and test day.				Nitrogen of urine, 24 hours.	Metabolism for 24 hours.	
							Carbohydrate.	Protein.	Fat.	Calories.		Basal, calories.	Total (basal plus 20 per cent), calories.
							C				N		M
	1921	yrs.		cm.	kg.		gm.	gm.	gm.		gm.		
A366687	Sept. 10	44	M	162	58	E	0	0	0	0	6.62	1,335	1,602
A373176	Oct. 28	53	"	168	110	D	60	60	100	1,422	8.92	1,885	2,260
A374395	" 19	15	"	157	34	D	17	40	83	1,006	9.03	1,084	1,302
A374229	Nov. 14	63	"	169	72	D	25	54	172	1,923	11.53	1,625	1,950
A378133	Dec. 13	43	"	183	79	D	33	52	200	2,216	10.00	1,768	2,122
A377198	" 6	31	"	173	56	D	23	37	140	1,548	6.72	1,243	1,492
A368030	Sept. 5	14	"	164	51	E	35	35	213	2,268	6.23	1,561	1,873
A377653	Dec. 6	51	"	162	46	D	21	29	120	1,321	4.87	1,094	1,313
A377262	Nov. 30	53	"	183	81	D	22	60	166	1,880	11.19	1,807	2,168
A371214	Sept. 14	30	"	174	66	E	34	45	196	2,147	7.56	1,793	2,152
A374275	Oct. 26	37	"	171	52	D	20	30	140	1,507	9.42	1,521	1,825
A376588†	Nov. 16	48	F	170	88	D	30	60	149	1,755	8.96	1,805	2,161
A375561†	" 9	31	"	163	39	D	14	10	86	902	5.45	1,025	1,230
A365349	Aug. 12	16	"	169	42	D	20	30	119	1,313	4.40	1,152	1,382
A370281	Sept. 13	14	M	157	33	D	14	10	87	903	3.90	962	1,154
A367133	" 13	23	"	170	42	D	14	10	87	903	4.37	1,080	1,297

\* E = epilepsy; D = diabetes.

† Mild infection as a complication; an acute afebrile nasopharyngitis.

The important data obtained in these experiments are presented in tabular form (Table I). The first case in the series was a patient with epilepsy, who had been fasting for 11 days. Although he received no food, a knowledge of the total metabolism and nitrogen excretion permitted the calculation of the metabolizing mixture. The ketogenic ratio of this mixture was 2.8:1 and accompanying this high ratio, acetone bodies appeared in appreciable quantities in both blood and urine. In the other



These results (15) were discussed at the last meeting of the American Society of Biological Chemists. They harmonize with the conclusions reported by Shaffer (11) at that meeting and apparently permit conclusions as follows:

#### CONCLUSIONS.

1. Certain assumptions, stated herein, are employed in the calculation of the composition of the mixture of food substances engaging in metabolism. Under the conditions of these experiments, provided these assumptions are tenable, the ratio between the ketogenic and the glucose molecules at which a clinically significant ketosis appears has a value of at least 2:1. A ratio of this value implies that every molecule of glucose is ketolytic for 2 molecules of acetoacetic acid.

2. The existence of infection lowers the ketogenic threshold so that significant ketogenesis may occur with lower ratios. Other factors, thus far undetermined, may also lower this threshold. It is advisable, therefore, in planing diets for diabetic patients to allow only such food mixtures as will avoid the 2:1 ratio by a safe margin.

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# THE RÔLE OF CYSTINE IN THE DIETARY PROPERTIES OF THE PROTEINS OF THE COW-PEA, *VIGNA* *SINENSIS*, AND OF THE FIELD PEA, *PISUM SATIVUM*.\*

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The large production of the cow-pea, the best known and most extensively grown leguminous crop of the southern states (1), and of the field pea, one of the most important legumes cultivated in the northern states and in Canada, makes the study of the nutritive value of their proteins of particular interest. Another consideration adds to the interest of this study. Botanically, the cow-pea is more closely related to the bean (*Phaseolus*) than to the pea (*Pisum*). On the basis of this relationship, it might be predicted that the proteins of the cow-pea would be limited in their nutritive value by the same factors which we have found in the case of the navy (2), lima (3), and adzuki (4) beans; namely, a deficiency in cystine and, in the case of the navy and lima beans, the need of cooking.

The experiments recorded in this paper show that these two limiting factors also apply to the proteins of the cow-pea. The field pea, on the other hand, is not limited in its nutritive properties by these factors, since satisfactory growth of young albino rats was obtained with the peas either raw or cooked, and without the addition of cystine in a diet adequate with reference to the other essential dietary factors.<sup>1</sup>

\* A preliminary report of this paper was presented at the Fifteenth Annual Meeting of the American Society of Biological Chemists held in Chicago, December 28 to 30, 1920 (cf. Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1921, xlv, p. xxv.)

<sup>1</sup> The inorganic constituents of the diet were furnished by the salt mixture of Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374.)

Some confusion of names has arisen both between the cow-pea, *Vigna sinensis*, and the field pea, *Pisum sativum*, and between the latter and the vetch, *Vicia sativa*, which is sometimes erroneously referred to as "peas." In the southern states the cow-pea is frequently called the field pea or simply pea. Furthermore, according to Dr. C. V. Piper,<sup>2</sup>

"The true pea (*Pisum sativum*) consists of two groups of varieties, the sugar or garden peas whose seeds wrinkle when dry, and the field peas which remain smooth and round when dry. The former are grown only for human food and eaten green; the latter are used largely for forage, particularly in Canada, and are therefore called Canada field peas. The split peas of the market are field peas. The vetch, *Vicia sativa*, is used as forage. Most varieties have dark colored seeds and are not used as human food. One variety with straw-colored seeds is, however, used sparingly after the manner of lentils for food."

*Nutritive Value of the Proteins of the Cow-Pea, Vigna sinensis.*

In the practical feeding of farm animals the cow-pea has been generally recognized as having a high feeding value when incorporated with other feedstuffs, in the general diet. Furthermore, it is considered a very palatable and nutritious human food. As far as the authors are aware, however, the nutritive value of the total proteins of the cow-pea when fed as the sole source of protein in the diet has not been investigated.

In feeding experiments with the isolated globulin *vignin*, the chief protein of the cow-pea, Osborne and Mendel (5) found it to have a relative nutritive value slightly greater than that of wheat gliadin. The proteins of the cow-pea have been chemically studied by Osborne and Campbell (6), who isolated three globulins, one of which very closely resembled phaseolin, the chief protein of the navy bean. Vignin, the chief globulin of the cow-pea, was later hydrolyzed (7) and the amino-acids were determined. The results of this hydrolysis show that, aside from cystine which was not determined, the amino-acids which are known to be nutritionally essential are present in this protein in amounts adequate

<sup>2</sup> By personal communication from Dr. C. V. Piper, Agrostologist in charge of Forage-Crop Investigation, Bureau of Plant Industry, United States Department of Agriculture.

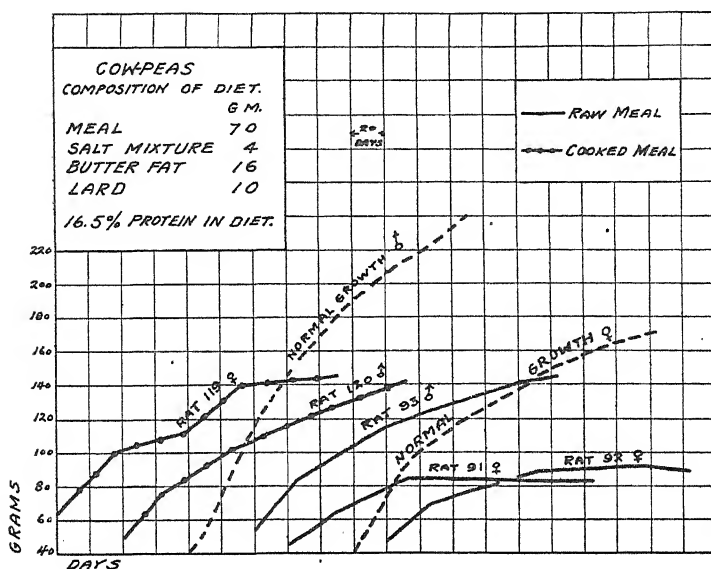


CHART 1.

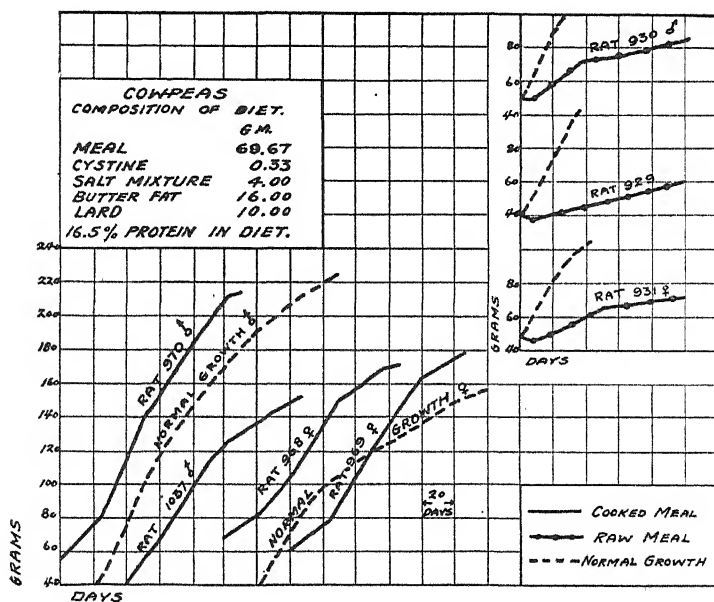


CHART 2.



to fulfill the requirements of normal nutrition. Although the percentage of tryptophane was not determined, it was shown to be present. The close relationship of the cow-pea to the beans of the genus *Phaseolus*, not only botanically, but also with reference to chemical and nutritive properties, is shown by the foregoing studies.

The cow-peas used in our experiments were of two varieties,<sup>3</sup> the Groit and the Brabham. The former has a ground color of buff, marbled with brown and thickly sprinkled with minute blue specks, while the latter variety is buff, marbled with brown. No difference was observed in the results obtained when either of the two varieties was used. The meal as used in the experimental diets contained 23.6 per cent of protein ( $N \times 6.25$ ). Growth was obtained at only one-third to two-thirds of the normal rate with either the raw or cooked meal when no cystine was added. Addition of cystine to the extent of 0.33 per cent of the diet produced little or no better results when the raw meal was used. However, when this same amount of cystine was added to the diet containing the cooked cow-pea meal growth was obtained at the normal rate. The composition of the diet and the results of these experiments are recorded on Charts 1 and 2.

*Nutritive Value of the Proteins of the Field Pea, Pisum sativum.*

The field pea has been extensively used in feeding farm animals. It was found by Murray (8) to be very efficient in a ration for milch cows, especially when the peas were mixed with oats, while Carlyle (9) found that the peas used alone as the grain part of a ration are no better than corn with respect to the amount of growth produced.

Pea meal has been shown by Osborne and Mendel (10) to supplement satisfactorily the deficiencies of corn proteins when fed in a diet containing 15.7 per cent of protein, 7.8 per cent of which was furnished by corn gluten.

<sup>3</sup> The cow-peas used in these experiments were furnished by the Bureau of Plant Industry, United States Department of Agriculture. A description of these seeds is given by W. J. Morse in *Farmers Bulls.* 1148 and 1153, United States Department of Agriculture.

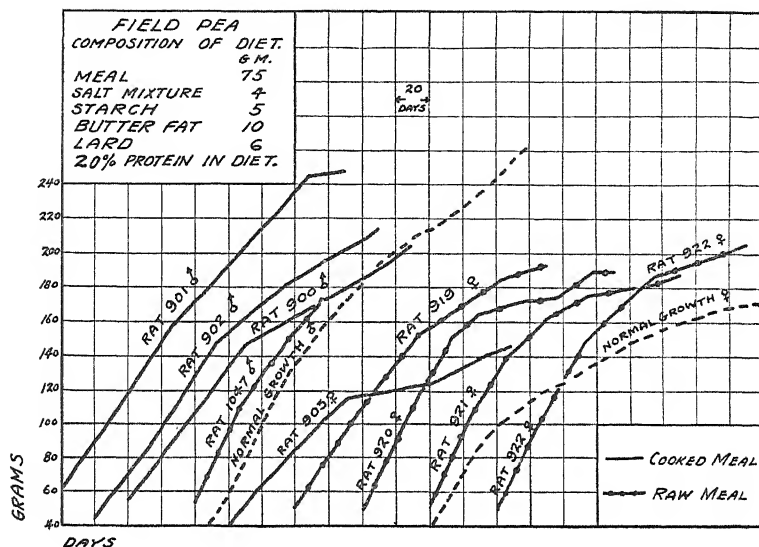


CHART 3.

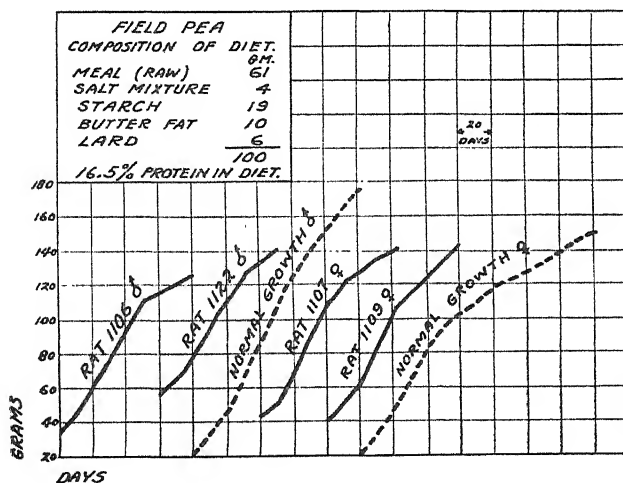


CHART 4.

McCollum, Simmonds, and Parsons (11), in an article on the "Dietary properties of the pea, *Vicia sativa*," describe feeding experiments conducted with rats, in which the seeds they used constituted the sole source of protein in an otherwise adequate diet. They obtained only slow growth with early stunting, from which they concluded that the "pea proteins are of very poor quality." It is to be noted, however, that the seeds they used were *Vicia sativa*, and not *Pisum sativum*.

Sure (12) has recently reported that in feeding experiments to determine whether proline is the growth-limiting factor in the proteins of *Vicia sativa*, which McCollum, Simmonds, and Parsons had previously shown to be nutritionally inadequate, he found several individuals in one lot of rats that grew at an almost normal rate. The explanation of these unexpected results was made clear when a new shipment of seeds which this particular lot of rats had been receiving were identified as *Pisum sativum*.

The peas used in our experiments were obtained in the open market as dried, smooth, yellow, split peas, and were identified as *Pisum sativum*,<sup>4</sup> the common field pea. When the meal obtained by grinding these peas was fed, either raw or cooked, to albino rats in a diet of which it constituted 75 per cent, equivalent to 20 per cent of protein, growth was obtained at the normal rate (Chart 3). Growth at practically the normal rate was also obtained when the field peas constituted 61 per cent of the diet, equivalent to 16.5 per cent of protein (Chart 4).

*Relative Efficiency of the Proteins of the Cow-pea and of Those of the Field Pea.*

Accurate records of the food intake were made, and the gain in weight per gram of protein ingested calculated. From Table I it will be seen that during the 10 week period the proteins of the field pea, and those of the cooked cow-pea, plus cystine were utilized equally well, while the proteins of the cow-pea, raw, plus cystine, or cooked without cystine, were less than half as well utilized as those of the field pea.

<sup>4</sup> The identification of these peas was made by Dr. W. W. Tracy, Sr., of the Office of Horticultural and Pomological Investigations, Bureau of Plant Industry, United States Department of Agriculture.

The results of these feeding experiments indicate a fundamental difference in the quality of the proteins of the cow-pea and those of the field pea, and show that the nutritive shortcomings of the proteins of the cow-pea are to be ascribed to the same causes as those found in the case of the beans of the genus *Phaseolus*; namely, a deficiency in cystine and a form of indigestibility which can be remedied by cooking. That this increase in the nutritive value brought about by cooking is to be ascribed to an improvement in digestibility has been shown in the case of the proteins of the navy bean, *Phaseolus vulgaris*, in a previous publication from this laboratory (13), by means of digestion studies *in vitro*.

TABLE I.  
*Average Gain of Body Weight per Gram of Ingested Protein.*

	Cow-pea diet.*			Field pea diet.*
	Cooked.	Raw + cystine.	Cooked + cystine.	Raw.
	gm.	gm.	gm.	gm.
Gain per gm. of protein ingested (4 week period).....	0.61	0.90	0.90	1.49
Gain per gm. of protein ingested (10 week period).....	0.45	0.43	1.01	1.01

\* 16.5 per cent protein in the diet.

#### SUMMARY.

The proteins of the cow-pea, *Vigna sinensis*, like those of the beans of the genus *Phaseolus*, are limited in their nutritive value by a deficiency of cystine and by an indigestibility which can be remedied by cooking. Only one-third to two-thirds of the normal rate of growth of albino rats was obtained on a diet in which 70 per cent of cow-pea meal, equivalent to 16.5 per cent of protein, furnished the sole source of protein.

Field pea (*Pisum sativum*) meal, fed at the same protein level, enabled rats to grow at a practically normal rate without the addition of cystine or cooking.

The proteins of the field pea and those of the cooked cow-pea plus cystine were equally efficient in promoting growth at a practically normal rate, while the proteins of the cow-pea raw, plus

cystine or cooked, without cystine, were less than half as well utilized as those of the field pea.

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## THE DETERMINATION OF MAGNESIUM IN BLOOD, PLASMA, AND SERUM.

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In 1920, I published the description of a method for the determination of magnesium in blood (1). This procedure, which was adapted for use on the filtrates obtained after the precipitation of calcium in whole blood, serum, or plasma by Lyman's (2) method, consisted essentially in the precipitation of magnesium ammonium phosphate and the nephelometric determination of the phosphate in this compound by the strychnine molybdate reagent which has of late years been used by numerous investigators for the quantitative determination of small amounts of phosphates.

A few months after the description of this method had been published, there appeared a paper by Bell and Doisy (3) describing a colorimetric method for the determination of small amounts of phosphate by means of molybdic acid which appeared to offer certain distinct advantages over the above mentioned nephelometric procedure.

An attempt to apply this colorimetric technique to the determination of magnesium ammonium phosphate has given uniformly good results, so that for some months I have entirely abandoned the use of the nephelometer for this work.<sup>1</sup>

<sup>1</sup> In the proceedings of the Sixteenth Annual Meeting of the American Society of Biological Chemists published in the February number of the Journal of Biological Chemistry, Briggs (Briggs, A. P., *J. Biol. Chem.*, 1922, 1, p. xlviii) has under the title of "Colorimetric methods for the determination of homogentisic acid and magnesium" published the following note: "Phosphomolybdic acid is especially susceptible to reduction by *p*-diphenols. Advantage of this reaction has been taken, first, to determine homogentisic acid in alcapton urine, and again to determine magnesium by the phosphorus content of the  $Mg\ NH_4\ PO_4$  precipitate." In view of the fact

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Magnesium determinations by the magnesium ammonium phosphate method can be made only on material free from calcium, and it is, therefore, the custom to use for this determination the filtrate obtained after the precipitation of calcium.

Much activity has been shown in the past few years in perfecting methods for the determination of blood calcium. The procedures so far proposed fall into two general classes: Those in which calcium oxalate is precipitated after the removal of protein, and those in which this precipitation is brought about in the presence of blood and serum proteins. The latter method of precipitation, which has recently been recommended by Kramer, Tisdall, and Howland (4) to the exclusion of procedures of the former type, seems destined to be extremely popular and I have, therefore, carried on a series of experiments to determine the possibility of using the magnesium method on the filtrates obtained from both the above types of precipitations.

As an example of a method involving precipitation in a protein-free medium I have used filtrates obtained from the determination of calcium by the method of Lyman (2), while to test the possibility of precipitation in a protein-containing liquid I have used the supernatant liquids obtained on the determination of calcium in serum by the procedure of Clark (5).

The determination of magnesium in the liquid remaining after the precipitation of calcium according to the procedure of Lyman is carried on in the following manner. If it is planned to use the supernatant liquid from the calcium analysis for the determination of magnesium it is best, before the removal of calcium is attempted, to evaporate an appropriate quantity (corresponding to from 1.5 to 2.0 cc. of blood or serum) of the trichloroacetic acid filtrate almost to dryness, about 10 cc. of distilled water are then added and in turn allowed to evaporate rapidly. This removes most of the trichloroacetic acid, and greatly facilitates the subsequent precipitation of magnesium. After the second evaporation about 10 cc. of water are added and the precipitation of calcium is accomplished according to the directions contained in Lyman's paper. The supernatant liquid removed from the calcium precipitate as well as the portion of ammonium oxalate solution used

that no details of his procedure are given by Briggs, I have felt it desirable to publish a description of the procedure for magnesium determinations which has been in use in the laboratory for the past 8 months.

for washing is transferred to a 100 cc. beaker and evaporated to a volume of 2 cc. To this residue is added 0.5 cc. of a 5 per cent solution of ammonium phosphate containing 5 cc. of concentrated ammonium hydroxide per liter and 2 drops of concentrated ammonium hydroxide. The beaker is then covered and allowed to stand not less than 10 hours. At the end of this time the contents of the beaker are transferred to a centrifuge tube which is centrifuged for about 5 minutes. The supernatant liquid is then syphoned off and the beaker and tube are washed with 5 cc. of a mixture of 1 part concentrated ammonium hydroxide (sp. gr. 0.9) and 2 parts of water. The wash liquid is placed in the tube and after centrifugation is removed from the precipitate by syphon. This process is repeated with two more portions of wash liquid. After the third portion has been removed the tube and beaker are washed with 5 cc. of 75 per cent alcohol containing 10 cc. of concentrated ammonium hydroxide per liter, and after removal by syphon of this portion of alcoholic ammonia the tube and beaker are allowed to stand in a warm place until the ammonia has evaporated.

The magnesium ammonium phosphate is then dissolved in 5 cc. of 0.1 N hydrochloric acid, and the solution so obtained transferred, with the aid of an additional 5 cc. of 0.1 N hydrochloric acid to a 25 cc. volumetric flask.

The remainder of the process consists essentially in the use of the technique described by Bell and Doisy (3) for the determination of inorganic phosphates in blood. To the solution contained in the volumetric flask are added 1 cc. of molybdic acid reagent, 2 cc. of hydroquinone reagent, and after an interval of 10 minutes 10 cc. of carbonate sulfite solution, the flasks are then filled to the mark with water, and after mixing the contents are read against an appropriate standard.

The most convenient standard is one prepared from magnesium ammonium phosphate dissolved in 0.1 N hydrochloric acid; which is of such concentration that 5 cc. contain 0.010 mg. of magnesium. 10 cc. of this standard are placed in a 25 cc. volumetric flask and treated with molybdic acid, hydroquinone, and sulfite carbonate mixture in the same manner and at the same time as the unknown.



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The determination of magnesium in the supernatant liquid remaining after the direct determination of calcium in serum by Clark's (5) method is as follows: 2 cc. of serum are placed in a conical centrifuge tube and to this is added with stirring 1 cc. of 3 per cent ammonium oxalate solution. After standing over night the tube is then centrifuged and the precipitated calcium oxalate collected at the bottom of the tube. The above constitutes the first step of the method of calcium determination advocated by Clark. 2 cc. of the supernatant liquid are pipetted into a 15 cc. centrifuge tube, and to this is added with stirring 0.5 cc.

TABLE I.  
*Recovery of Magnesium Added to Serum, Plasma, and Blood.*

Nature of material.	Magnesium in original material per 100 cc.	Magnesium added per 100 cc.	Magnesium found per 100 cc.	Method of calcium precipita- tion.
Blood, human.....	1.6	0.5	2.00	Lyman.
“ “ .....	1.6	1.0	2.71	“
“ “ .....	1.6	2.0	3.80	“
Serum, “ .....	2.0	0.3	2.32	“
“ “ .....	2.0	0.5	2.50	“
“ “ .....	2.0	0.9	2.87	“
“ “ .....	2.0	0.5	2.48	Clark.
“ “ .....	2.0	1.0	3.06	“
“ “ .....	2.0	2.0	3.94	“
“ “ .....	2.0	2.5	4.52	“
Plasma, “ .....	2.2	1.0	3.21	“
Serum, dog .....	1.9	0.5	2.43	“
“ “ .....	1.9	0.8	2.70	“
“ “ .....	1.9	1.0	2.92	“

of a 5 per cent solution of ammonium phosphate containing 5 cc. of concentrated ammonium hydroxide per liter. The tube after being allowed to stand over night, is centrifuged, the supernatant liquid is syphoned off, and the remaining steps of the determination are carried on as directed above.

The method of precipitation and colorimetric determination outlined above have been carried out on pure solutions containing from 0.02 to 0.10 mg. of magnesium with an average recovery of 96 per cent. In Table I are collected the figures obtained on various samples of blood plasma and serum before and after the

addition of known quantities of magnesium. From these results it is apparent that the method can be relied on to give results within an error of 5 per cent.

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# THE RELATION BETWEEN THE ENDOGENOUS CATABOLISM AND THE NON-PROTEIN CONSTITUENTS OF THE TISSUES.

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Folin's theory of protein metabolism has received general recognition among writers in nutrition, and many of the deductions formulated by Folin from observations of the effect of a variable protein intake upon the composition of the urine, have been strikingly verified by later experimental work. However, other theoretical deductions, intimately related to his theory, do not occupy such a secure position at the present time. This seems to be true particularly of the conception of the endogenous protein catabolism as defined and described by Folin in 1905.<sup>1</sup>

Opposed to the theory of Folin that there is a constant basal catabolism of protein, commensurate with the protein requirement for the maintenance of life, may be placed the more recent theories of Sherman<sup>2</sup> and of Osborne and Mendel.<sup>3</sup>

Sherman's theory was advanced primarily to explain the fact that the nitrogen of mixtures of amino-acids, so incomplete as not to cover the maintenance requirements, may nevertheless be utilized, often to a large extent, while even single nitrogenous compounds, such as individual amino-acids or some of the ammonium salts may have a favorable influence on the nitrogen balance of animals. In accounting for these facts, the conception of the reversible reaction has been put forward in relation to the hydrolysis of tissue proteins and the deamination of the amino-acids

<sup>1</sup> Folin, O., *Am. J. Physiol.*, 1905, xiii, 117.

<sup>2</sup> Sherman, H. C., *J. Biol. Chem.*, 1920, xli, 97.

<sup>3</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 328.

thus formed. It is supposed that the hydrolysis of tissue proteins, constituting the initial step in the endogenous catabolism, may be checked or even reversed by an increase in the concentration of amino-acids in the tissues during digestion and absorption. Thus, any amino-acid will function in the maintenance of body protein, though the more complete the mixture of amino-acids coming from the intestinal tract the more marked will this effect be, since each amino-acid could be expected to check the hydrolysis only at the point at which that particular amino-acid would be liberated from the protein molecule. According to this theory the utilization of the nitrogen of incomplete mixtures of amino-acids is only apparent. The real effect is simply an inhibition or retardation of the endogenous catabolism. In a similar way, it is assumed that the apparent utilization of ammonium salts is in fact only a retardation of another reversible reaction, in this case that of the deamination of amino-acids.

The theory of Osborne and Mendel of the endogenous metabolism was advanced specifically to explain why certain proteins, notably the gliadin of wheat, may supply the nitrogenous needs of an animal in maintenance, and yet be entirely inadequate for the purposes of growth. It was their idea that the maintenance protein requirement may in reality be a requirement for definite amino-acids that may serve special physiological functions; for example, serving as the material for the synthesis of the active constituents of the secretions of the endocrine glands. The body protein, on a nitrogen-free diet, would thus undergo degradation only because by this method some essential amino-acids are liberated. The theory is consistent with the view that the endogenous catabolism would be greater on a non-nitrogenous diet than on a nitrogenous one, since in the latter case the nitrogenous requirements for hormone precursors would be covered by food protein, not body protein; hence the hydrolysis of body protein would cease, and the endogenous catabolism would be reduced to the degradation of the hormones themselves in the course of metabolism. The endogenous nitrogen would hence be reduced on a diet containing complete proteins, possibly to only a small fraction of its value on a nitrogen-free diet.

While it is of scientific interest to know why the endogenous catabolism occurs and what factors affect it, it is also of practical

interest to know whether this disintegration of cellular nitrogenous compounds continues at the same rate, whether the animal is getting protein in its ration or not. The endogenous catabolism of nitrogen seems to be related to the maintenance requirement of protein. If Folin's theory is correct in its entirety, the maintenance requirement of protein is a constant basal requirement, to which must be added the requirements for growth or milk production. In the formulation of feeding standards for farm animals, this assumption is always made, the requirement of protein for any particular type of production being added to a basal requirement, as determined by maintenance experiments of one type or another.

If the theory of Sherman is correct, or the theory of Osborne and Mendel, no such basal requirement of protein exists, and no simple additive relation between a maintenance requirement and a requirement of protein for growth, or any other purpose, could be assumed. In fact, it may very well be, according to these theories, that the endogenous catabolism is largely or entirely suppressed when an animal is receiving protein in its food.

Also in experiments designed to measure the nutritive value, or the so called "biological value," of proteins for maintenance or growth, the question of the reality of a constant basal catabolism of protein arises. The often cited work of Thomas on the relative value of the proteins of different foods for the maintenance of nitrogen equilibrium in the human organism, is based upon the assumption of a basal catabolism of protein, commensurate with the excretion of urinary nitrogen on a nitrogen-free diet. While Thomas' experimental results are subject to severe criticism, the theory upon which his calculations of "biological values" are based has not been successfully attacked. On the other hand, the recent method proposed by Osborne and Mendel<sup>4</sup> of measuring the value of different proteins for growth by the ratio of gain secured to protein consumed, ignores a possible basal catabolism of tissue protein calling for a constant maintenance requirement of dietary protein. If such a constant requirement is being met under conditions of growth, only that fraction of the protein intake

<sup>4</sup> Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxvii, 223.

over and above the maintenance quota would be related to the growth secured.

The theoretical and practical importance of the endogenous protein catabolism, or more accurately, the endogenous catabolism of nitrogenous compounds, is sufficiently great to justify experimental investigation. Folin's classical investigations were concerned with the composition of the urine, when the protein metabolism was reduced to the minimum tissue catabolism by feeding a very low protein diet of starch and cream. It was the hope of the authors in planning this experiment that the effect of feeding a similar ration on the concentration of non-protein nitrogenous compounds in the tissues of rats might throw additional light on this obscure type of catabolism.

#### EXPERIMENTAL.

Twelve male white rats, weighing from about 200 to 300 gm. each, were the subjects of the first experiment. They were all placed upon a ration of milk and dog biscuit when received from The Wistar Institute. Six of them were taken off this ration and killed and analyzed after being fasted for 19 to 26 hours. The remaining six were changed to a synthetic ration containing minimal amounts of nitrogen. Of these, one was killed after being kept 24 hours on the experimental diet, one was killed after 48 hours, two after 11 days, and two after 22 days. The ration contained 76 per cent of starch, 10 per cent of butter fat, 8 per cent of sucrose, and 3 per cent each of salts (Osborne and Mendel's mixture) and agar; also a small amount of Osborne and Wakeman's Fraction II from yeast as a source of vitamine B. On analysis, this ration was found to contain 0.82 mg. of nitrogen per gm.

The rats were killed with ether and the intestinal tract was cleaned out with distilled water, and the entire carcass was then passed twice through a meat grinder. The ground material was immediately extracted with boiling water slightly acidified with acetic acid (0.5 cc. of glacial acetic acid per liter). When the first portion of water was added the mixture was quickly brought to boiling to prevent enzymic changes. In all cases the tissues were exposed to boiling water within 15 minutes of the death of the animal, so that it may be fairly assumed that the chances for

postmortem changes were minimal. Each carcass was extracted eight times with five times its weight of boiling nitrogen-free water, and was digested on the steam bath for 15 to 30 minutes after each addition of water. The extracts were filtered through funnels containing a loose plug of glass-wool, covered by two or three layers of cheese-cloth. The filtrates were collected and made up to a definite volume, and aliquots taken for analysis for total non-protein nitrogen, ammonia, amino nitrogen, urea, and creatine. Occasionally, the total soluble nitrogen was also determined.

Protein was removed from one set of aliquots by concentration at a low temperature, and precipitation with 10 volumes of 95 per cent alcohol. Amino nitrogen was determined in aliquots from these filtrates after removal of ammonia by rendering slightly alkaline and distilling *in vacuo* as recommended by Van Slyke.

Urea and ammonia were determined by aeration of concentrated aliquots made alkaline with potassium carbonate, half of the aliquots having been previously incubated with urease according to the method of Van Slyke and Cullen. Creatine was determined in other aliquots by concentration, hydrolysis in approximately normal sulfuric acid solution by immersion for 3 hours in boiling water, and colorimetric determination of the creatinine. The solutions after hydrolysis were exactly neutralized, made up to volume, and filtered. Aliquots of 10 cc. from the filtrates were immediately transferred to 100 cc. flasks and treated with 15 cc. of saturated picric acid solution, previously tested for development of color with alkali, and 2 cc. of a 10 per cent solution of NaOH. After 10 minutes standing the flasks were diluted to the mark, shaken, and read in the colorimeter against a solution prepared in a similar way from a creatinine standard containing 1 mg. per cc. The standard was set at 15 mm.

To determine the effect of the type of nitrogenous catabolism upon the total sulfur and total nitrogen of the tissues, and the non-protein sulfur and nitrogen, seven rats were taken. Four of the rats were taken from a normal ration, fasted for about 20 hours (with the exception of Rat 1226, which was not fasted), and then killed with ether and prepared for analysis. Three of the rats were placed upon the same non-protein ration used in the preceding experiment, except for the salt mixture, which in this



case was prepared by substituting phosphoric for sulfuric acid. It was, therefore, nearly sulfur-free as well as being very low in nitrogen. After subsisting for 10 days on this ration, the rats were killed and prepared for analysis.

Since it was desired to make direct determinations of total sulfur and nitrogen on the tissues of these rats, more care was taken in the preparation of the carcasses for analysis. Each carcass was passed through a sausage mill three or four times and then through a Straub laboratory mill (Model F4) about four or five times, after which treatment it was in a finely divided and homogeneous condition. A small amount of a 10 per cent alcoholic solution of thymol was mixed with each sample, which was then transferred to a jar and placed on ice until analyzed. Samples were weighed out for total nitrogen and total sulfur, the latter being determined by Halverson's modification of the Benedict method,<sup>5</sup> using the Denis oxidation mixture.<sup>6</sup> 50 gm. samples were extracted with hot acidified water, concentrated, and precipitated with alcohol, as in the preceding experiment, and used for the determination of non-protein nitrogen and sulfur, the latter being determined by the Denis modification<sup>6</sup> of the Benedict method for sulfur in urine.

#### EXPERIMENTAL RESULTS.

For the two rats in the first experiment kept for 11 days on the nitrogen-free ration, and for the two kept for 22 days on this ration, daily collections of urine and feces were made and were analyzed for nitrogen, in order to determine how soon the rat reached its minimum endogenous catabolism of nitrogen and how constantly the minimum was maintained from day to day. The details of the method used in this laboratory for metabolism work on rats will be described in a later paper. The results of these determinations are given in Tables I and II.

It is evident that on a nitrogen-free diet the rat very quickly reaches a minimum nitrogen excretion, which then very slowly decreases as the body weight decreases. All these rats (as well as the two rats fed only for 24 and 48 hours, respectively, on this ration) were fasted for some 20 hours before collection of urine

<sup>5</sup> Halverson, J. O., *J. Am. Chem. Soc.*, 1919, xli, 1494.

<sup>6</sup> Denis, W., *J. Biol. Chem.*, 1910, viii, 401.

and feces. After 24 hours of feeding, the urinary nitrogen had reached a level with Rats 915A and 915B, which was maintained throughout the subsequent 10 days. This level was equivalent to the daily excretion of 16.6 and 15.1 mg. of urinary nitrogen, respectively, per 100 gm. of rat. With Rats 926A and 926B, this low level of urinary nitrogen was not reached until the latter part of the feeding period. From the 4th to the 11th day of feeding, the average urinary excretion of nitrogen was 21.1 mg.

TABLE I.

*Daily Excretion of Nitrogen of Rats 915A and 915B on a Nitrogen-Free Ration.*

Day.	Rat 915A.				Rat 915B.			
	Weight.	Food.	Urine N.	Fecal N.	Weight.	Food.	Urine N.	Fecal N.
	gm.	gm.	mg.	mg.	gm.	gm.	mg.	mg.
0	327				297			
1		14.0	98			14.0	112	21
2		14.0	53	41		14.0	47	31
3		14.0	67	31		14.0	56	30
4	321	14.0	49	42	295	14.0	49	27
5		16.0	46	38		15.6	33	42
6		15.0	48	48		12.9	40	25
7	314	13.1	47	25	290	13.2	47	17
8	309	13.4	58		286	12.1	37	28
9	307	9.3	62		287	10.7	39	26
10	300	9.6	59	45	275	9.7	48	31
11			41	32			51	11
12	302				272			
Total.....			628	415*			559	289

\* Assuming an average excretion of nitrogen on those days for which the fecal determination was lost.

per day per 100 gm. of body weight for Rat 926A, and 21.3 gm. for No. 926B. For the last 8 days of feeding, the averages were 16.6 and 17.0 mg., respectively. This delay in reaching a comparable level of endogenous catabolism may be due to the fact that Rats 926A and 926B ate less of the nitrogen-free ration per day than Rats 915A and 915B.

The results of the analysis of the fasted and fed rats are given in Table III in mg. per 100 gm. of body weight, and in Table IV in percentage of the total non-protein nitrogen.

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The few determinations of the total soluble nitrogen that were made were quite variable and cannot be interpreted as indicating any effect of the non-protein ration. The total non-protein nitro-

TABLE II.  
*Daily Excretion of Nitrogen of Rats 926A and 926B on a Nitrogen-Free Ration.*

Day.	Rat 926A.				Rat 926B.			
	Weight.	Food.	Urine N.	Fecal N.	Weight.	Food.	Urine N.	Fecal N.
	gm.	gm.	mg.	mg.	gm.	gm.	mg.	mg.
0	317				240			
1		14.8	161	43		11.3	113	34
2		11.9	62			12.7	59	33
3		11.0	64	16		12.5	58	36
4	308	11.6	55	60	235	9.6	52	25
5		11.7	60	31		12.0	58	10
6		12.0	58	51		12.0	60	50
7	301	11.3	44	30	231	12.9	50	33
8		12.5	90	19		12.3	43	14
9		11.2	70	34		12.0	44	28
10	290	12.8	57	33	223	10.6	41	19
11	285	11.6	65	26	223	12.7	41	25
12	289	13.0	56	24	219	12.4	31	25
13	284	13.0	46	55	212	10.1	52	30
14	281	12.6	51	19	209	9.9	45	22
15	276	14	50	64	213	14	41	22
16	273	13	43	36	212	13	31	30
17		12	57			12	41	31
18		12	46	28		12	51	12
19		12	31	23		12	28	25
20	264	12	40	35	195	12	29	24
21		12	49	31		12	30	34
22		12	39	26		12	28	
23	261				202			
Total.....			1,294	752†			1,026	590

\* This figure represents the combined food residues for the last 8 days of the feeding period.

† Assuming an average excretion of nitrogen on those days for which the fecal determination was lost.

gen was much more constant. In general, the values for the rats in normal nutritive condition run higher than those for the rats fed for varying periods on the low nitrogen ration, the average

value for the former being 272 mg. as compared with an average value of 247 mg. for the latter. We would hardly be justified in attaching any significance to this average difference because of the variation in this value within the two groups of rats.

The ammonia figures are so variable as to render their interpretation impossible. The values for the amino nitrogen were re-

TABLE III.

*Non-Protein Nitrogenous Constituents of the Tissues of Rats in Normal Nutritive Condition, and in a Condition in Which the Nitrogenous Catabolism Has Been Reduced to the Endogenous Level.\**

Rat.	Length of fasting or feeding period.	Weight of rat.	Forms of nitrogen per 100 gm. of rat.					Creatine per 100 gm.
			Total soluble.	Non-protein.	Ammonia.	Amino.	Urea.	
Fasted rats.								
	hrs.	gm.	mg.	mg.	mg.	mg.	mg.	mg.
912	19	288		273		54		
921	19	238		292	19	51	20	219
1006	19	275	984	276	19	51	27	224
1014	19	185	797	241	15	46	36	217
1027	23	208		254	16	44	31	212
1004	26	297	1,072	296	28	54	20	210
Fed rats.								
1013	24	220	850	197	15	39	46	162
1025	48	190		243	14	48	21	185
	days							
915A	11	302		261	23	49	12	193
915B	11	272		268	23	53	8	188
926A	22	261	1,134	222	15	43	25	179
926B	22	202	996	289	16	42	16	185

\* Results are expressed in mg. per 100 gm. of body weight.

markably constant in both groups of rats, possibly running slightly higher with the fasted than with the fed rats. With one exception the percentages of amino nitrogen (Table IV) for all rats fell within the narrow range of 17 to 20. This constancy in the amino nitrogen concentration of the tissues regardless of the type or the intensity of the catabolism of nitrogenous material, is one of the most interesting results of this investigation.

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The urea concentration of the tissues shows considerable variation, particularly in the group of rats killed after varying periods on the protein-free ration. In this group the urea was relatively high for the rat fed only 24 hours, lower for the rat fed 48 hours, and, with the exception of the value for Rat 926A, lower still for the rats fed for longer periods of time. Except for the unaccountably high figure for this rat, it appears probable that the urea

TABLE IV.

*Non-Protein Nitrogenous Constituents of the Tissues of Rats in Normal Nutritive Condition, and in a Condition in Which the Nitrogenous Catabolism Has Been Reduced to the Endogenous Level.\**

Rat.	Length of feeding or fasting period.	Weight of rat.	Total non-protein nitrogen per 100 gm.	Distribution of non-protein nitrogen.			
				Ammonia.	Amino.	Urea.	Creatine.
Fasted rats.							
	<i>hrs.</i>	<i>gm.</i>	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
912	19	288	273		19.8		
921	19	238	292	6.5	17.5	6.8	24.1
1006	19	275	276	6.9	18.5	9.8	26.1
1014	19	185	241	6.2	19.9	14.9	28.9
1027	23	208	254	6.3	17.3	12.2	26.8
1004	26	297	296	9.5	18.2	6.7	22.8
Fed rats.							
1013	24	220	197	7.6	19.8	23.3	26.4
1025	48	190	243	5.8	19.8	8.6	24.4
	<i>days</i>						
915A	11	302	261	8.8	18.8	4.6	23.8
915B	11	272	268	8.6	19.8	3.0	22.5
926A	22	261	222	6.7	19.4	11.3	25.9
926B	22	202	289	5.5	14.5	5.5	20.5

\*Results are expressed in per cent of the total non-protein nitrogen.

concentration of the tissues is reduced as the exogenous protein catabolism is abolished, but that it never entirely disappears. One can, therefore, conclude that urea is an end-product of the endogenous as well as of the exogenous type of catabolism.

The values obtained for creatine indicate clearly a slight though distinct lowering of the concentration of this substance in the tissues as the result of the change in the type of protein catabolism produced by feeding a ration free from protein. All the values

obtained for the fed rats were lower than those for the rats in a normal nutritive condition in the postabsorptive period. The average creatine content of the fasted rats was 216 mg. per 100 gm. of body weight, while the average for the fed rats was 182 mg., representing an average reduction of 16 per cent. This experimental result is in general agreement with the results of Myers and Fine,<sup>7</sup> who observed a marked reduction in the creatine con-

TABLE V.

*Total and Non-Protein Nitrogen and Sulfur of the Tissues of Rats in Normal Nutritive Condition, and in a Condition in Which the Nitrogenous Catabolism Has Been Reduced to the Endogenous Level.\**

Rat.	Body weight.	Total S.	Total N.	Total N: total S.	Non-protein S.	Non-protein N.	Non-protein N: non-protein S.
Rats on non-protein ration for 10 days.							
	gm.	mg.	mg.		mg.	mg.	
1222A	116	264	3,103	11.7	35.9	275	7.7
1222B	138	262	2,990	11.4	31.6	210	6.6
1222C	127	245	2,818	11.5	31.0	212	6.8
Rats on normal ration.							
1226†	164	248	2,917	11.7	35.4	297	8.4
1227A	118	251	2,899	11.6	33.7	220	6.5
1227B	137	266	3,160	11.9	34.7	218	6.3
1227C	222	270	3,201	11.8	34.1	238	7.0

\* Results are expressed in mg. per 100 gm. of body weight.

† This rat was not fasted before being killed.

tent of rabbit muscle as the result of continued carbohydrate feeding.

The results obtained on the seven rats analyzed for total and non-protein nitrogen and sulfur are given in Table V. No effect of the 10 days subsistence on a nitrogen- and sulfur-free ration can be observed on any of the values determined. The ratio of total nitrogen to total sulfur in the bodies of these rats was remarkably constant, ranging from 11.4 to 11.9, to 1. In the protein-free extracts, the ratio of nitrogen to sulfur was much less, ranging from 6.3 to 8.4, to 1. Neither of these ratios shows any significant differences between the two groups of rats.

<sup>7</sup> Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 305.

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In two cases, aliquots of the extracts, after precipitation with alcohol, were tested gravimetrically for inorganic sulfates, with inconstant results. A test for conjugated (etheral) sulfates also gave no positive indications. Evidently the occurrence of oxidized forms of sulfur in the tissues is confined to mere traces, when considered on the body as a whole. Recently, Denis<sup>8</sup> has shown that the sulfate content of blood is normally extremely small, requiring special methods for its determination.

It is to be noted in particular that the concentration of non-protein nitrogen in these seven rats, analyzed several hours or even days after death, is not significantly different from the values given in Table III for the rats analyzed immediately after etherization. This is some indication of the absence of any considerable postmortem changes in the experimental material and enhances somewhat the confidence that may be placed in the significance of the analytical results.

### DISCUSSION.

The approximate constancy of the concentration of the total non-protein nitrogen and sulfur and of the amino nitrogen of the tissues, regardless of the type or the intensity of the catabolic processes resulting in nitrogenous or sulfur-containing end-products, would seem to be a matter of significance in formulating a theory of the endogenous catabolism. In explaining a similar situation as related to the amino-acid concentration of the tissues as influenced by fasting, Van Slyke and Meyer<sup>9</sup> put forward two possibilities as to the origin and function of the free amino-acids of the tissues: first, that the amino-acids might serve as a reserve energy supply or as a reserve of tissue-building material; and, second, that their presence in the tissues might be dependent simply upon the fact that they are intermediate steps in the construction and the breakdown of tissue proteins, originating either from absorbed food products or autolyzing tissue protein. From the fact that starvation does not decrease the concentration of amino-acids in the tissues, these investigators conclude that the second possibility is correct.

<sup>8</sup> Denis, W., *J. Biol. Chem.*, 1921, xlix, 311.

<sup>9</sup> Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913-14, xvi, 231.

However, from the fact that the concentration of amino-acids in the tissues is not diminished on continued feeding with a non-protein ration, which may be presumed to reduce the protein catabolism to a minimum, while affording no building materials for an anabolism of protein, it does not seem probable that the presence of amino-acids in the tissues is merely incidental to protein metabolism. Furthermore, it seems to be true that intermediary products of metabolism do not normally accumulate to any considerable extent in the tissues, as witness the absence of demonstrable amounts of the hydroxy- and keto-acids that must be produced in the breakdown of glucose, fatty acids, and amino-acids; nor do even the end-products of metabolism accumulate normally to any great extent.

It is fair to presume, therefore, that the free amino-acids of the tissues are not functioning as reserve material, nor are they present merely as intermediary steps in the synthesis and disintegration of protein, but that they are performing some distinct and important function in the life of the tissues, since such an effective mechanism exists for maintaining their concentration constant. Their remarkable constancy in the muscle fluids of migrating salmon is also significant in this connection.<sup>10</sup> Their possible function in the maintenance of osmotic pressure within the cell,<sup>11</sup> and particularly within the nucleus, which seems to be free from inorganic electrolytes, may also be cited.

The data obtained in this investigation also indicate that the total non-protein nitrogen and sulfur are not definitely affected by the type or intensity of the protein metabolism. Probably most of the substances contributing to the non-protein fraction of the tissue nitrogen and sulfur are performing some specific functions in metabolism. The universal presence of creatine in vertebrate muscle, the fact that it is not an obligate stage in the catabolism of arginine or any other known amino-acid, its differential occurrence in different tissues, and its extremely small concentration in the blood as compared to its concentration in the tissues, constitutes strong presumptive evidence that it is serving a function in tissue metabolism. Similarly with carnosine. This substance is a dipeptide containing an amino-acid,  $\beta$ -alanine, not thus

<sup>10</sup> Greene, C. H., *J. Biol. Chem.*, 1919, xxxix, 457.

<sup>11</sup> Collip, J. B., *J. Biol. Chem.*, 1920, xlii, 227.



far found in proteins, and is extremely resistant to tissue proteases,<sup>12</sup> both facts arguing for a purposive synthesis in muscle cells. In the same category may be placed the recently discovered autoxidizable dipeptide of Hopkins,<sup>13</sup> consisting of cysteine and glutamic acid. Apparently this compound performs important functions in the chemical dynamics of the cell. The constancy of the concentration of non-protein sulfur in the tissues, regardless of the intensity of catabolism, is consistent with this view.

The relation of the endogenous catabolism to these compounds—and there are probably many others that may be so classed—is an interesting subject of speculation concerning which little definite information is available. Being soluble and diffusible, and, as a class, less stable to oxidation than the cell proteins themselves, one might suppose that their loss from the tissues by purely physical means, or their destruction by catabolic agents, would be more constant, certainly more inevitable, than the loss of the proteins in the cell.

If the non-protein nitrogenous substances are continually being lost to the tissues as an incidental consequence of metabolism, it is conceivable that the losses may be replaced from a variety of sources, even from such simple substances as ammonium salts. Possibly any one of the amino-acids may be used in the synthesis of one or more of these compounds, either as an obligate or a facultative precursor. Some of them may require some one particular precursor, such as tryptophane, tyrosine, cystine, or histidine, so that such amino-acids are indispensable to the maintenance of life. Some of the amino-acids that are essential in the synthesis of cell proteins, may not be obligate precursors of any of these nitrogenous extractives, and hence would not be essential for maintenance.

It is conceivable that the endogenous catabolism of Folin consists essentially of the continual and constant breaking down of those non-protein nitrogenous compounds of the tissues, so essential to their proper functioning. When no protein is being consumed the losses thus incurred may be replaced by the destruction of cell proteins. In such a case, it is possible that the nitrogen thus degraded can be used *without waste* in replacing these essential

<sup>12</sup> Baumann, L., and Ingvaldsen, T., *J. Biol. Chem.*, 1918, xxxv, 263.

<sup>13</sup> Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

non-protein substances, so that the urinary nitrogen of an animal on a nitrogen-free diet and a sufficient energy intake would represent the minimum endogenous catabolism. When protein is being consumed in quantity the destruction of body protein may cease entirely and the integrity of the tissues be maintained from exogenous sources. In replacing the endogenous losses, food proteins may be expected to be utilized in general to a greater extent than in growth.

The theory thus easily and simply explains one of the most puzzling features of the many experiments recorded in the literature relative to the amino-acid requirements of maintenance. It has been shown clearly and repeatedly that the requirements of the body for amino-acids to maintain the integrity of the tissues continually being impaired by the endogenous catabolism, are different from the requirements for the synthesis of proteins, quantitatively and possibly qualitatively. Also that individual amino-acids or even some of the ammonium salts, groups of amino-acids insufficient to cover completely the maintenance requirement, and proteins such as clupeine, gelatin, and zein, so incomplete in their amino-acid structure that, as the sole source of nitrogen, they cannot maintain life, are nevertheless utilized in the repair of the tissues, sometimes to a very complete extent. The explanation of McCollum<sup>14</sup> that "the processes of cellular catabolism and repair do not involve the destruction and resynthesis of an entire protein molecule," implies that the disintegration of protein to some extent is an essential feature of the endogenous catabolism. Similarly, the somewhat clearer statement of the same conclusion by Steenbock and Gross:<sup>15</sup> "In endogenous protein catabolism the destruction of all amino-acids is not in proportion to the extent in which the individual amino-acids occur in the disintegrating protein molecule." It is difficult to conceive of the tissue proteins undergoing only partial hydrolysis, always retaining some of the amino-acids, such as lysine, in the intact portion. A much less awkward explanation of the facts would be to dispense entirely with the conception that protein disintegration is an essential part of cell activity.

<sup>14</sup> McCollum, E. V., and Steenbock, H., *Univ. Wisconsin Agric. Exp. Station, Research Bull.* 21, 1912, 79.

<sup>15</sup> Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1913, xxxvi, 285.

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That the endogenous catabolism may not be essentially a catabolism of protein is indicated by the distribution of the different forms of nitrogen in the urine when the diet is sufficient in energy but practically nitrogen-free. Folin's experiments on the composition of human urine on a normal diet and on a starch and cream diet, show clearly that when the urinary nitrogen is derived mainly from the catabolism of food protein, about 90 per cent of it occurs as urea plus ammonia, some 3 to 4 per cent as creatinine, 1 to 2 per cent as uric acid, and about 5 per cent in undetermined compounds. On the other hand, when the diet is practically nitrogen-free, only about 70 per cent occurs as urea-ammonia, 2 to 3 per cent as uric acid, 14 to 18 per cent as creatinine, and 7 to 12 per cent or even more as undetermined substances. Substantially the same results were obtained by McCollum and Hoagland<sup>16</sup> on the pig, fed for several weeks on a diet of starch, an alkaline salt mixture, and agar-agar.

If the corresponding quantities of endogenous nitrogen be deducted from the total nitrogen, urea nitrogen, and ammonia nitrogen excreted on the protein-containing dietary, it may be calculated that the exogenous catabolism of proteins results in an excretion of nitrogen of which some 95 per cent is in the form of urea plus ammonia.

Similarly, if the endogenous catabolism is accelerated under conditions which obviously induce a considerable destruction of cellular protein, as in fasting, the composition of the urine approaches that resulting from exogenous protein catabolism.

The same distinction between the sulfur-containing products of protein catabolism, and of the minimum endogenous catabolism, exists as between the nitrogen-containing metabolites. Evidently protein metabolism, whether of the exogenous or the endogenous variety, yields sulfate sulfur entirely, while 25 per cent or more of the sulfur-containing metabolites of the minimum endogenous catabolism (*non-protein catabolism*) is in the form of unoxidized sulfur. The ratio of nitrogen to sulfur in the urine when the nitrogenous catabolism is reduced to the lowest level by the feeding of nitrogen-free nutrients only, is 10 to 12 to 1 from Folin's data. On the other hand, during fasting, when most of the nitrogenous catabolism is undoubtedly protein in nature, this ratio is 15 to 17 to 1, more nearly approaching the ratio of nitrogen to sulfur in cellular proteins.

If the endogenous catabolism represents protein disintegration, as is generally assumed, one would expect the elimination of the various nitrogenous end-products of this catabolism to proceed at a fairly uniform rate or to be interdependent in their variations. However, this does not seem to be the case. The creatinine elimination is remarkably uniform from day to day and from hour to hour, and does not seem to be appreciably affected by any normal condition of nutrition. In marked contrast with this is the wide and unaccountable fluctuation to which the uric acid excretion is subjected, not only from day to day, but from hour to hour. As Folin's has

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<sup>16</sup> McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913-14, xvi, 299, 317, 321.

said of the endogenous uric acid in the urine: "The metabolic processes that determine the uric acid excretion may therefore be said to be in relatively unstable equilibrium." Also, many investigators have shown that food free from purine precursors, particularly protein food, may markedly raise the level of endogenous uric acid excretion. Recently, Lewis, Dunn, and Doisy<sup>17</sup> have shown that proteins and amino-acids may increase the hourly elimination of uric acid 100 or 200 per cent with no perceptible effect on the excretion of creatinine. The independence of the excretion of these two metabolites in pathological states has also been clearly shown.

In their study of the endogenous metabolism of pigs, McCollum and Hoagland<sup>16</sup> have demonstrated that the minimum nitrogenous catabolism, to which the pig is reduced on a diet of starch and a salt mixture of a potentially alkaline character, may be accelerated in different ways in response to different stimuli. When a mineral acid is added to the basal diet the endogenous catabolism is accelerated entirely by an increase in ammonia excretion, the excretion of urea and creatinine being unaffected. When benzoic acid is added to the basal ration in small to moderate amounts, the total endogenous catabolism may not be accelerated at all. However, a portion of the nitrogen which, in the absence of benzoic acid, would appear as urea, is diverted to the synthesis of hippuric acid, no other appreciable effect on metabolism resulting. The urea nitrogen cannot apparently be decreased to a lower level than about 20 per cent of the total, a synthesis of glycocoll from other sources occurring with increasing doses of benzoic acid. With the ingestion of excessive amounts of benzoic acid, there is a marked acceleration in the total nitrogenous catabolism with an increased excretion of ammonia. Furthermore, Lewis and Karr<sup>18</sup> have shown that the ingestion of sodium benzoate affects the hourly excretion of uric acid, causing a marked diminution (50 to 70 per cent) in the first 4 hours after benzoate administration, while no effect on creatinine was noted.

The evidence cited to the effect that the rate of elimination of those end-products of the endogenous catabolism that are not obviously interconvertible in the body, may be varied independently of each other, may be interpreted as meaning that the precursors of these metabolites are to be found among the non-protein compounds of tissues, each of which probably has some specific function to perform and is catabolized at varying rates in response to variations in the function it serves. The evidence is not readily explained by the ordinary view that the precursors of the endogenous metabolites are united firmly in a protein molecule.

The facts concerning the excretion of creatine in the urine are also consistent with the theory that the minimum endogenous catabolism of nitrogenous compounds, is essentially not a catab-

<sup>17</sup> Lewis, H. B., Dunn, M. S., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 9.

<sup>18</sup> Lewis, H. B., and Karr, W. G., *J. Biol. Chem.*, 1916, xxv, 13.

olism of protein, but of non-protein substances. Aside from the occurrence of creatine in the urine, induced obviously by exogenous protein metabolism,<sup>19</sup> it always appears in the urine under conditions of an accelerated endogenous metabolism associated with obvious tissue destruction, as in fasting, experimental diabetes, diabetes mellitus, hyperthyroidism, muscular dystrophy, etc. The facts of the normal or pathological occurrence of creatinuria may be satisfactorily explained by the theory that creatine is a typical end-product of endogenous protein metabolism, and the fact that it does not occur in the urine when an animal is reduced to its minimum endogenous catabolism, is satisfactorily explained by the theory that this basal nitrogenous catabolism does not essentially involve protein disintegration.<sup>20</sup> The theory is fortified by the large amount of evidence in the literature demonstrating a total independence in the excretion of creatinine, indicative, possibly, of the endogenous non-protein catabolism, and of creatine, indicative of an endogenous protein catabolism, when not of exogenous origin. The association of a reduced excretion of creatinine with a constant creatinuria, as in muscular dystrophy, diabetes mellitus, hyperthyroidism, and similar pathological conditions involving cachexia and reduced muscular efficiency, is particularly convincing confirmation of the theory advanced.

<sup>19</sup> Possibly the failure of Rose, Dimmitt, and Bartlett (Rose, W. C., Dimmitt, J. S., and Bartlett, H. L., *J. Biol. Chem.*, 1918, xxxiv, 601) to confirm the findings of Denis and Minot as to the relation between high protein feeding and the occurrence of creatinuria in women (Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1917, xxxi, 561) might be due to the fact that the high protein diets of Denis and Minot contained 50 gm. per day of gelatin, a protein very rich in arginine, containing almost twice as much as the proteins of milk, which were used, together with eggs, in both experiments in the high protein diets. In the high protein diets of Rose and associates, gelatin was not included.

<sup>20</sup> The apparently erratic occurrence of creatine in the urine of fasting pigs, reported by Steenbock and Gross (Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxvi, 265), is accounted for by this theory. Those pigs excreting, during fasting, amounts of nitrogen in the urine commensurate with the minimum endogenous catabolism, exhibited no creatinuria, while those pigs excreting amounts of total nitrogen in considerable excess of this level, in every case exhibited creatinuria in proportion to the daily excretion of urinary nitrogen. The following data on the five fasting pigs investigated by Steenbock and Gross, and earlier by McCollum and

The theory advanced here as to the nature of the minimum endogenous catabolism of nitrogenous compounds thus appears to reconcile the data on the composition of the urine on a non-protein diet, upon which Folin's theory was based, the available data as to the utilization of nitrogenous compounds in the repair processes of the tissues, upon which McCollum's theory was based, the data on the amino-acid requirements for maintenance as compared to those for growth, upon which the theories of Sherman and of Osborne and Mendel were based, and, at the same time, the data presented in this paper on the general constancy in the concentration of the non-protein nitrogenous constituents of the tissues following the suppression of the exogenous metabolism of protein.

*Significance of the Reduction in Creatine.*

The values obtained for the concentration of creatine in the tissues of the rats maintained for varying periods of time on a practically nitrogen-free ration were all lower than those obtained for the rats taken from a normal ration. In the latter rats, it may be presumed that both exogenous and endogenous catabolism of nitrogenous compounds were operating, while in the former case only the endogenous catabolism was occurring. It seems fair to presume, therefore, that the reduction in the concentration of creatine was due to the absence of an exogenous protein catabolism.

It would appear that most of the creatine in the tissues is present as an integral part of the protoplasmic mechanism, performing some function in the life of the cells. A relatively small and variable fraction of the creatine in the tissues would seem to be derived

Steenbock (McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912-13, xiii, 209) demonstrate the point made:

Weight of pig.	Days of fast (inclusive).	Total nitrogen in urine.	Creatinine nitrogen in urine.	Creatine in urine.
		<i>gm.</i>	<i>per cent</i>	<i>gm.</i>
85	7-15	3.11	9.17	0.000
51	4-6	5.47	2.67	0.393
55	4-5	10.50	1.12	0.836
59	3-5	9.25	1.94	0.412
77	6-9	2.97	10.11	0.000

from the catabolism of dietary protein, probably representing an intermediate state in the catabolism of arginine and possibly other amino-acids. If the intake of dietary protein is excessive, and particularly if the protein is rich in arginine (and possibly other creatine precursors), some of the creatine, produced normally only as an intermediary product, finds its way into the urine. Thus far, for reasons not at all obvious, creatinuria of the type just mentioned has only been observed with women and children.

The experimental results on creatine reported in this paper and the explanation offered to account for them, are in entire agreement with the data reported by Thompson,<sup>21</sup> who obtained a small though distinct increase in the percentage of creatine in rabbit muscle following the intravenous injection of arginine. The somewhat similar work of Myers and Fine<sup>22</sup> also falls in line with the data we have obtained, and may be explained in a like fashion.

Myers and Fine analyzed the muscles of a fairly large number of rats, some of which had been fed on edestin, a protein containing 14 per cent of arginine, and others on casein, a protein containing only about 4 per cent of this amino-acid. A slight difference in creatine content of the two groups of rats was noted, the edestin rats having the greater concentration. While the authors themselves do not attach any significance to the difference observed, and while the experiment is generally referred to as an indecisive one, a statistical analysis of the data put them in a different light. The eleven casein rats had an average content of  $0.4582 \pm 0.0012$  per cent creatine in the muscle. The seven edestin rats had an average percentage of  $0.4701 \pm 0.0011$ . The average difference is  $0.0119 \pm 0.0016$ , being thus more than seven times its probable error. According to ordinary statistical standards it may be presumed to possess a high degree of significance, and to indicate in no uncertain way that the edestin feeding has increased the creatine content of the muscle, probably on account of the high percentage of arginine in edestin. If the individual results be arranged in the order of a decreasing content of creatine, one casein rat will be found to rank third, having the same percentage as two edestin rats, another casein rat ranks eighth, and the other

<sup>21</sup> Thompson, W. H., *J. Physiol.*, 1917, li, p. ii.

<sup>22</sup> Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 389.

nine casein rats occupy the nine last places in the list. This can hardly be considered a random distribution.

It seems fairly certain, therefore, that tissue creatine is in part of exogenous origin, the concentration of exogenous creatine, representing an intermediary product in protein catabolism, depending primarily upon the intensity of catabolism of exogenous arginine and possibly of other creatine precursors.





# THE VOLATILE OIL OF MENTHA AQUATICA LINNÉ, AND A NOTE ON THE OCCURRENCE OF PULEGONE.

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In a recent article on the possible mode of production of peppermint oil<sup>1</sup> in the living plant, the writer pointed out the need of a conclusive investigation of the oil of *Mentha aquatica* Linné, which is now regarded as one parent of peppermint. Several statements are to be found in the literature concerning this oil.<sup>2</sup> In the original article on which these statements were based, the following information is given:<sup>3</sup> two distillates of "Bergamot mint" from Florida were examined by Schimmel and Company, the first from immature plants had an ester content of approximately 11 per cent, the second from frozen leaves had an ester content of approximately 39 per cent, "calculated as linalyl acetate." Of the second it was recorded that "in consequence of the higher ester content, the odor of linalyl acetate was here even more pronounced than in the previous oil." The following analysis is submitted in verification of these empirical observations.

## *Material.*

The oil of *Mentha aquatica* Linné employed in this investigation had been grown and distilled by the Wisconsin Pharmaceutical Experiment Station at Madison in 1919 and was very kindly placed at the writer's disposal by the Director. It was a practically colorless oil and possessed a very pleasant characteristic odor. 200 cc. (183 gm.) were used for the following experiments.

<sup>1</sup> Kremers, R. E., *J. Biol. Chem.*, 1922, 1, 31.

<sup>2</sup> Gildemeister, E., *Die Aetherischen Oele*, Leipsic, 2nd edition, 1910, i, 369, 528.

<sup>3</sup> Semiannual Report of Schimmel and Company, 1904, i, 95.

*Constants.*

The following values were found for the original oil:

$d_{24}$	=	0.916	Ester	=	73.82
$n_{24}$	=	1.4582	Alcohol as ester	=	58.00
$[\alpha]_D^{24}$	=	-7.48°	Ester No. after acetyl	=	224.0
Acid No.	=	7.84	Total alcohol	=	61.6
Ester No.	=	210.93	Free alcohol	=	3.6

*Aldehyde.*

A qualitative test for aldehyde revealed the presence of this type of compound. The oil was twice shaken with 30 cc. of 50 per cent bisulfite solution. The first reaction mixture became noticeably warm. The residual oil was washed with water, and the combined aqueous solutions were extracted with ether. The regeneration of the aldehyde was brought about by the addition of 1 mol of potassium hydroxide. The substance thus liberated resinified at once, and an immediate extraction with ether failed to give any material capable of reacting with semicarbazide.

*Free Acid.*

The oil from which the aldehyde had been removed was extracted with sodium carbonate solution. The acids thus extracted were not sufficient for identification.

*Saponification.*

The oil, 173 gm., was now saponified by heating on the water bath for 1 hour with 50 gm. of potassium hydroxide dissolved in 750 cc. of 65 per cent alcohol. The addition of the alkali caused the mixture to turn dark red, which resulted in a dark oil being precipitated by the addition of water at the end of the reaction. The oil was washed with water until neutral, and then dried over anhydrous sodium sulfate. The alcohol was largely distilled off from the alkaline aqueous solution before the latter was concentrated on the water bath to recover the potassium salts.

*Fractionation.*

The recovered oil was fractioned *in vacuo*, the second run resulting in the series:

Fraction.	Boiling point. <sub>30</sub> °	Weight.	$n_{24}$
	°C.	gm.	
1	-100	8	1.375
2	100-05	85	
3	105-35	9	1.4665
4	135+	15	Residue.
Total.....		117	

Fraction 2 was redistilled through a Vigreux column with the following results:

B. P.<sub>21</sub> = 95.5-97.5°C.; weight = 73 gm.;  $n_{D24}$  = 1.4610;  $d_{24}$  = 0.858;  $[\alpha]_D^{24}$  = -18.94°.

Recorded for linalool:<sup>4</sup>

B. P.<sub>14</sub> = 86-87°C.;  $d_{20}$  = 0.8622;  $n_{20}$  = 1.46108;  $[\alpha]_D$  up to -20°.

*Linalool Phenylurethane.*

A phenylurethane was prepared from fraction B.P. 95.5-97.5° by the method given for linalool in the Semiannual Report of Schimmel and Company for 1902.<sup>5</sup> The reaction proceeded as described. A slight brownish coloration could not be removed from this preparation, hence the melting point was a trifle low. M.P. found, 64-65°C.; recorded for linalool phenylurethane, 65-66°C. Therefore, linalool was the alcohol present.

*Identification of Combined Acid.*

As previously mentioned, the alcohol was largely distilled off from the alkaline saponification mixture. As no oil was precipitated by this process, the residual aqueous solution was evaporated on the water bath without extraction. When concentrated to about 150 cc., a resin separated which was filtered out. The

<sup>4</sup> Tiemann, F., *Ber. chem. Ges.*, 1898, xxxi, 834.

<sup>5</sup> Semiannual Report of Schimmel and Company, 1902, pt. 2, 70.

solution was then acidified with 44 gm. of sulfuric acid, previously diluted to 100 cc. The volatile acids were recovered by steam distillation. The distillate was neutralized with sodium hydroxide, phenolphthalein as indicator, and again evaporated. When the residue had become a syrup it was set aside and became a solid mass of crystals after standing some time. Qualitative tests on this salt indicated acetic acid.

In order to prove conclusively the presence of this acid, the preparation of a crystalline derivative was undertaken. For this purpose the anhydrous salt was first secured by heating a sample to constant weight at 120°C.; 10 gm. lost 4.02 gm. of water. Calculated on the basis of anhydrous sodium acetate, this loss corresponds to 3.06 molecules of water of crystallization: crystallized from water, sodium acetate retains 3 H<sub>2</sub>O.

This material was used for the preparation of *p*-nitrobenzyl and phenacyl esters according to the methods of Reid,<sup>6</sup> but without success. In the first instance mixtures with variable melting points were obtained; in the second only oily products resulted. Some impurity must have been present in slight amount. This may have been a valeric acid, as an odor of that character developed when the salt was kept in a sulfuric acid desiccator.

The desired result was finally obtained as follows: 4.4 gm. of salt were mixed with 3 cc. of phosphorus trichloride in an acetylation flask and heated on a water bath for 15 minutes. 5 gm. of aniline were added, the reaction mixture was somewhat broken up and again heated for a few minutes. The cooled product was extracted with 50 cc. of water; the residue was then recrystallized from 100 cc. of hot water. Characteristic plates of acetanilide were obtained; M.P. 114°C. No depression resulted after mixing with pure acetanilide.

#### CONCLUSIONS.

The oil of *Mentha aquatica* Linné distilled from normal mature plants, grown with necessary cultural precautions, has been shown to consist largely of linalool acetate. Smaller quantities of

<sup>6</sup>Reid, E. E., *J. Am. Chem. Soc.*, 1917, xxxix, 124. Lyman, J. A., and Reid, E. E., *J. Am. Chem. Soc.*, 1917, xxxix, 701. Lyons, E., and Reid, E. E., *J. Am. Chem. Soc.*, 1917, xxxix, 1727. Rather, J. B., and Reid, E. E., *J. Am. Chem. Soc.*, 1919, xli, 75.

another ester, of free linalool, of a free acid, and of a very unstable aldehyde were also present.

The elaboration of the oil by the plant can be thought of as following the same course as that outlined for *Mentha spicata* Hudson but stopping with the esterification of linalool.

*Occurrence of Pulegone in Mentha piperita.*

Methyl-1-cyclohexanone-3 and acetone are the products of hydrolysis of pulegone. The occurrence of the first in the "Vorlauf" of the cohobated oil of American peppermint and of the second in the cohobated aqueous distillate led to the assumption that pulegone was their forerunner in the elaboration of the oil by the plant.<sup>1</sup> Inasmuch as some menthone fraction, partially freed from menthol by freezing, of the oil which had given these results<sup>7</sup> was still available a search was made for pulegone.

The oil was first assayed by the neutral sulfite method—original volume was 10 cc., final volume was 9 cc.; the loss was 1.0 cc. corresponding to 10 volumes per cent of pulegone. The separation of the ketone was then undertaken. The oil was diluted with one-fourth of its volume of alcohol and set aside with a solution of 50 gm. of sodium sulfite in 100 cc. of water. The mixture was frequently agitated. After 6 weeks but few crystals had formed. The bisulfite liquor was separated and treated, together with the crystals, with 1 molecular equivalent of potassium hydroxide.

The regenerated ketone was extracted with ether, dried over sodium sulfate, and fractionated. B.P.<sub>17</sub> = 105–110°C.;  $n_D^{20}$  = 1.484.

The semicarbazone, prepared in the usual way and recrystallized from alcohol, melted at 169–170°.

Pulegone is, therefore, a constituent of the cohobated oil of peppermint, as had been previously suggested.

<sup>7</sup> Kremers, R. E., *J. Am. Pharm. Assn.*, 1921, x, 834.



## THE HYDROGEN ION CONCENTRATION OF HUMAN FECES.\*

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Many observations have been reported on the acidity or alkalinity of feces, but only a few attempts have been made actually to define the limits of fecal reaction. Matthes (9) found that feces were alkaline to cochineal, methyl orange, and lacmoid; and acid to phenolphthalein, rosolic acid, and curcuma. This was confirmed by Hemmeter (4). It would place the reaction somewhere between the limits pH 5.5 and 7.5. The general conclusion was drawn from this and other early work with indicators that the usual reaction was approximately neutral but was subject to fluctuations in either direction.

The first actual measurement of the hydrogen ion concentration of feces was made by Howe and Hawk (6), who used the electrometric method. The reaction of the material examined by them was always alkaline, varying with different subjects and conditions from pH 7.01 to 8.77.

Nelson and Williams (13) devised a method for measuring the hydrogen ion concentration of feces colorimetrically. In their experiments the material was usually acid, in only one case out of thirty did the pH rise above 7.0. As a rule it varied between 5.0 and 6.0 and in some cases went below 5.0.

Rettger and Cheplin (14) using a modification of the Nelson and Williams method likewise found the reaction to be acid,<sup>1</sup> varying between pH 5.0 and 6.8.

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<sup>1</sup> Rettger and Cheplin (14), p. 108.



*Factors Influencing the Fecal Reaction.*

The factors responsible for the variation in the fecal reaction may be divided into three classes; *viz.*, dietary, physiological, and biological.

The rôle of diet in causing fluctuations in the reaction of human excreta has been the subject of considerable debate. Matthes claimed that diet was not a factor in causing changes in the reaction of feces. Howe and Hawk found that pronounced variations in diet such as changes from high to low protein intake or fasting were accompanied by no consistent fluctuations in the reaction. The drinking of large quantities of water, however, appeared to produce a slight increase in alkalinity. McClendon, Myers, Culligan, and Gydesen (10) in connection with their investigation of the reaction of contents of the ilium state:

"The mineral content of the food does not seem to be a factor in determining the pH since the acidity of the intestinal contents is greater on a diet of carrots that yield a basic ash than on a diet of oats that yield an acid ash."

On the other hand, Hecht (3) claims that sugars cause with infants the elimination of an acid stool.<sup>2</sup> Schmidt and Strasburger (16) attribute changes in fecal reaction chiefly to biological causes and claim that it can be regulated indirectly by dietary control of the intestinal flora. In regard to the direct influence of diet they state that with good digestion a mixed diet gives a reaction fluctuating about the neutral point. A preponderance of carbohydrates or fat produces an acid stool while with a heavy meat intake the reaction, though variable, tends to be alkaline. Mother's milk causes the excretion of acid feces while cow's milk changes the reaction to alkaline.

The question of infants' stools has received special attention and the change of reaction with the change in diet from mother's milk to cow's milk has been noted by several. Muller (12) claims the alkalinity with cow's milk to be due to the putrefaction of the increased protein. Schlossmann (15) attributes the change not to the mother's or cow's milk as such, but to the change in the protein-fat ratio. A ratio of 1 part of protein to 3 or more of

<sup>2</sup> Hecht (3), p. 15.

fat (as in mother's milk) gives an acid stool while a decrease in the fat to a ratio of 1 to 1 or less gives an alkaline stool.

The effect of the physiological factors has received but little mention. It has been quite conclusively shown by investigators, notably McClendon and his coworkers, that the contents of the small intestine are acid. When a change in reaction occurs, it probably takes place after the material enters the large intestine where under the influence of the alkaline secretions of that part of the gut it tends to become alkaline. Muller states that, due to this fact, the outside of a stool which comes into direct contact with the mucous membrane is more alkaline than the inside. References of this kind are, however, few, and although it is quite generally recognized that acid stools accompany diarrhea and alkaline ones follow constipation the effects have been ascribed to bacterial action rather than to the intestinal secretions themselves.

Thus the biological factor has usually been accorded a preeminent place in determining fecal reaction. The widespread usage of sour milk which has followed the enunciation of Metchnikoff's theories of longevity has accompanied the general belief that the inoculation of the human intestine with acidophilic organisms caused a reduction of the putrefactive bacteria through the production of an acid medium inhibitive to their growth. The fallacy of this contention has been demonstrated by Rettger and his coworkers (14) who found no increase in the acidity of feces when the flora had been changed to one consisting of over 90 per cent (*Bacillus acidophilus*<sup>1</sup>). Other than this, actual experimental evidence of the influence of the bacterial flora on the fecal reaction appears to be nil and the unquestioned acceptance of the assumption, admittedly plausible though it be, that fecal reaction is controlled by biological factors must be considered hazardous.

The present work was undertaken for the purpose of securing data of a quantitative nature on the normal reaction of human feces from apparently healthy subjects on mixed diets. The effect of varying the retention of the material in the intestine and the influence on the fecal reaction of inoculating the intestine with acid-forming bacteria has also been studied.

## EXPERIMENTAL.

*Methods.*

All determinations of the hydrogen ion concentration were made electrometrically, the general technique and apparatus described by Clark (2) being used. The cell was modified by replacing the three-way cock used for admitting the sample and the hydrogen by a one-way cock with the entrance tube bent upwards. This change was advisable because the nature of the material handled made it necessary to introduce it through the large opening used to hold the stopper and electrode. The modified cell was also easier to clean and sterilize. The hydrogen was obtained from a tank and was purified by passing it through solutions of mercuric chloride, alkaline potassium permanganate, and dilute sulfuric acid. Originally it was passed over the heated filament of a tungsten lamp as advocated by Clark, but this was shown to be unnecessary with the particular gas used which apparently was free from oxygen.

No attempt was made to run the measurements at constant temperature beyond having the apparatus in an air bath to protect it from drafts and temperature changes during individual determinations. The temperature of the bath was always noted and the appropriate correction made.

Saturated calomel half-elements were used and a Weston cell which was frequently checked against one recently tested by the Bureau of Standards.

The electrodes were of gold-plated platinum or solid gold and were freshly platinized for each determination.

The whole procedure was frequently checked by determining the reactions of buffer solutions of known values.

The following procedure was adopted for measuring the hydrogen ion concentration of fecal material: A composite sample of the stool was thoroughly macerated with enough water to form a thin paste. Several analyses showed these pastes to contain about 4 per cent solid material, which is approximately the consistency of the intestinal contents as they enter the large intestine. A few cc. of this paste were poured into the hydrogen electrode vessel through the large opening, the bore of the three-way cock used to make connection with the calomel cell having been previously filled with liquid. The vessel was then connected on the one side with the vessel connecting it with the calomel cell and on the other

with the hydrogen purification train. The stopper holding the electrode was placed loosely in the opening and hydrogen admitted in a fairly rapid stream. The vessel was tilted at such an angle that the hydrogen passed over the surface of and did not bubble through the liquid. Gas was allowed to flow through for several minutes to replace all of the air and the stopper was forced into place while hydrogen was still entering the cell, thus preventing the advent of air. The stop-cock through which the hydrogen had been admitted was then closed and the tube disconnected. The vessel was finally rocked until equilibrium was attained, connection between it and the calomel cell established through saturated KCl solution by opening the proper stop-cock, and the electromotive force measured at once. As a rule measurements were made in duplicate with different electrodes, the rocking and reading being repeated until the values became constant.

From the standpoint of accuracy the advantages of this procedure over that of testing material for "alkalinity" or "acidity"

TABLE I.

*Comparison of the Reaction of Feces as Determined with Litmus Paper and Electrometrically.*

pH	Reaction to litmus.	pH	Reaction to litmus.	pH	Reaction to litmus.
6.2	Acid.	7.0	Alkaline.	7.4	Neutral.
6.6	Neutral.	7.0	"	7.4	Alkaline.
6.7	"	7.1	"	7.5	"
6.8	Alkaline.	7.1	"	7.8	"
6.9	"	7.3	"	7.9	Neutral.

by means of indicator papers are, of course, obvious and call for no discussion. Since the qualitative test with litmus paper has afforded the basis for much of the speculation indulged in on the subject of fecal reaction and still is the standard test, some idea of its reliability is of interest. The reaction to litmus paper of a large number of samples was observed and the results are recorded in Table I together with the pH values as found by the electrometric method. Sensitive red and blue paper was used. The strips were moistened with distilled water, the fecal suspension applied to one side of the paper, and the change in color observed on the other side.

It will be seen that while there is general agreement between the two methods, a large percentage of the results with the litmus paper are inaccurate. In some cases a comparatively high degree

of acidity is recorded as a one plus alkalinity. By using a series of tests a fairly reliable picture may be obtained, but single determinations are not to be depended upon and individual variations in a series may entirely escape detection. No quantitative value is to be given this test and it is impossible to say that a sample is "more" or "less" acid or alkaline than another from results obtained with it.

*Fecal Reaction of Apparently Healthy Men on a Mixed Diet.*

The results of the examination of the feces of two apparently healthy men on mixed diets are given in Table II. The figures are from mixed samples of the whole stools.

These pictures are to be regarded as normal. When a series of observations is made over a long period of time occasional values are obtained which are outside this range. They are generally isolated instances, the reaction returning promptly to the normal range within 24 hours. They are apparently produced by some temporary irritation of the intestine which, while producing no

TABLE II.

*Normal Fecal Reaction of Human Subjects on Mixed Diets.*

Subject A.....	7.2	7.1	7.2	7.4	7.1	6.9	7.2
" B.....	7.2	7.3	7.3	7.5	7.1	7.5	7.1

unusual sensation on the part of the subject, still shows itself in the fecal reaction.

From these results as well as from the examination of many stools of other individuals *the normal fecal reaction appears to vary between pH 7.0 and 7.5*. This is in agreement with the original value of Howe and Hawk. It is an interesting coincidence that the results obtained by the electrometric method agree with each other but do not check those obtained by the colorimetric method. The conclusion seems to be justified that the type of method used determines the result. Examinations kindly made by Dr. Nelson using his technique on samples being tested simultaneously by the electrometric method in the course of this work showed perfect agreement. Although this was done on only one or two samples it makes the above conclusion appear to be untenable. More work must be done before final judgment can be passed.

No attempt was made to control the diets of the subjects under examination so no positive statements can be made regarding the actual influence of the dietary factor. No pronounced direct effect on the fecal reaction of such changes in diet as occur ordinarily has been observed in this work. Variations in diet that tend to produce constipation or looseness of the bowels do exert indirect influences but the results are probably due to physiological rather than dietary causes.

*Influence of Physiological Factors on the Fecal Reaction.*

Under this head will be considered the difference in fecal reaction due to incomplete neutralization of the acid contents of the small intestine or the opposite effect; *i.e.*, the secretion of an excess of alkali.

The acid material passed into the large intestine may fail to be neutralized because of too rapid passage through the large intestine as in diarrhea, or by the failure of the large intestine to secrete sufficient alkali completely to neutralize the acid admitted to it. In either case an acid stool results. An alkaline stool may occur after prolonged exposure of the material to the alkaline secretions of the large intestine as in constipation or by the secretion of unusually large amounts of fluid under the stimulus of medication.

Under normal conditions and in the absence of complicating factors such as bacterial activity it may be assumed that the reaction of the contents of the large intestine changes progressively from the acidity of the contents of the small intestine to the alkalinity of the secretions as the material passes from the cecum to the rectum. The variation in reaction throughout the mass of that portion ordinarily passed in a single defecation should be comparatively small since it has already passed through the length of the large intestine and been exposed to its action for several hours. Nevertheless, small differences between the two ends of the stool would be anticipated.

In Table III are given results with Subject B, the samples being taken from the extreme ends of the stools, care being taken, of course, to get cross-sections of them and not an undue portion of the outside which would be more alkaline than the inside. The material in the first portions excreted (Series a) was held in the

colon approximately 24 hours longer than the Series b portion of the preceding stool. The difference in reaction between the Series a sample of one stool and the Series b sample of the preceding one should represent that due to retention in the colon for the interval between defecations. For convenience in comparing them the Series b values have all been advanced in the table to fall under the Series a values to which they should correspond. The results show the passage through the last portion of the colon actually is normally accompanied by a small increase in alkalinity. The unusual difference in values between the second Series b value and its corresponding Series a reading is presumably one of those abnormalities referred to above. Occasionally stools are encountered which are of normal consistency in their first portions but of unusual softness in the last portions passed. They may even be fluid. In such cases the desire to defecate is usually

TABLE III.  
*Change in Reaction of Feces during Passage through Colon.*

Series a.....	7.5	7.5	7.5	7.6	7.6	7.7	7.6
" b.....	7.4	6.7	7.2	7.5	7.4	7.6	7.3
Difference.....	0.1	0.8	0.3	0.1	0.2	0.1	0.3

more pronounced than at other times though one movement may be sufficient to give relief and there may be no further signs of diarrhea. The difference in reaction between the ends of such a stool will, however, be extraordinarily large, amounting in one case to pH 1.3. Usually the difference approximates 0.2 to 0.3 pH.

The influence of other agencies of this group was studied by making observations on the changes in the reaction of feces under the influence of various laxatives, in diarrhea, and in constipation. As pointed out above, the more rapid the passage of material through the large intestine the greater the tendency for its reaction to remain acid. A diarrheal stool should, therefore, be acid. This is normally true providing the stimulus producing increased peristalsis does not likewise cause increased secretion. In this case the effect of the former factor may be overcome to a greater or less degree and the final picture be the resultant of these two opposing tendencies.

Fig. 1 shows the results of a series of daily observations on Subject B during which period various laxatives were taken as indicated. During one part of the time the subject suffered from digestive disturbances including both diarrhea and constipation. The following cathartics were used: Magnesium oxide, phenolphthalein, physiological salt solution, castor oil, powdered aloes, powdered senna, and sulfur.

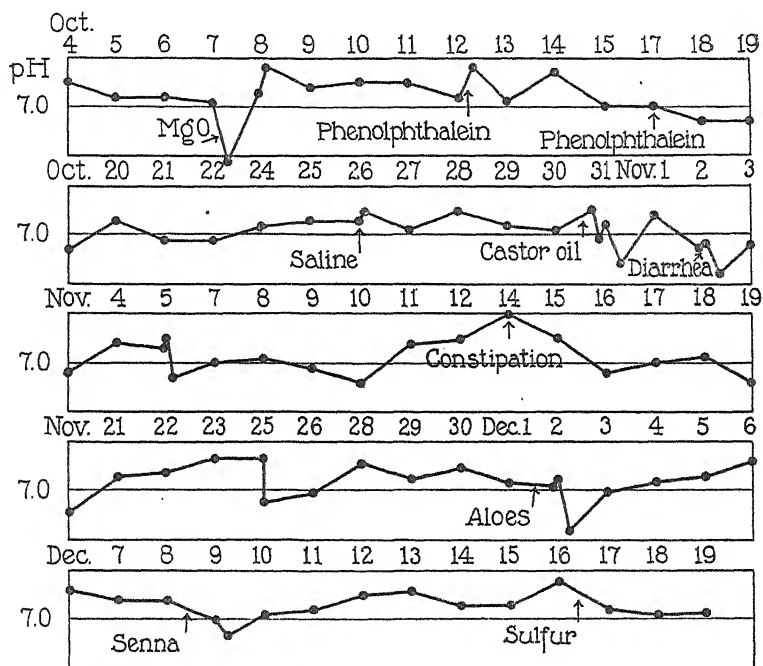


FIG. 1. Influence of cathartics on the fecal reaction. The ordinates in each case vary from 6.5 to 7.5.

*Magnesium Oxide.*—Aubert (1) first advanced the suggestion that saline purgatives act through stimulation of the nerve supply of the colon with the production of an increase in both the motive and secretory processes of the large intestine. This theory has been substantiated experimentally by MacCallum (8), Hertz, Cook, and Schlesinger (5), and others. Apparently the action is more thorough than with other agents, a complete evacuation of the whole large intestine being obtained. Magnesium oxide has



a twofold interest in the present work owing to its being both an alkali and a laxative.

The experiment was started on October 4, 1921. After 4 days of normal fecal reactions, 1 gm. of MgO was taken after each of three meals during October 7. 4 hours after the first dose,  $\frac{1}{2}$  hour after the second, and 6 hours after a normal stool had been passed defecation took place yielding a stool of the consistency of a thin paste which had a reaction corresponding to a pH 5.9. This was one of the most acid stools encountered during the whole course of the work. 16 hours later a soft but not liquid stool having a pH of 7.3 was passed. 3 hours later or 24 hours after the first dose of the medicine a third stool having a pH of 7.7 was obtained. Subsequent stools were normal in reaction.

The interesting point about this experiment is that the initial effect of the administration of magnesium oxide is the production of a distinctly acid stool, the effect of the alkali not being apparent for 24 hours after the first dose was taken. This coincides with the statement<sup>3</sup> of Hertz and coworkers (5, 7) that salts administered 1 day are not excreted until the following day although the laxative action is produced much sooner. It is supposed that the alkalinity is due to the direct neutralization of a part of the acid from the stomach and not to a stimulation of secretion. It should also be noted that the stool was acid in spite of a supposed stimulation of the secretory processes.

*Phenolphthalein.*—After a period of 4 days of normal fecal reaction 0.25 gm. of phenolphthalein was taken after breakfast. About 3 hours later a small, formed stool with a marked alkaline reaction of pH 7.8 was passed. There is some question as to its being the result of the medication, as no further disturbance occurred and the usual stool on the following day was normal. After 5 days another dose of 0.5 gm. of phenolphthalein was taken with the results that on the 2 following days soft, unformed, and acid stools were passed. With one exception the fecal pH was subnormal for several days. It is possible that factors other than the phenolphthalein were operative in producing these results although the appearance of the feces was normal in other respects and the subject suffered no unusual sensations. It is claimed that phenolphthalein stimulates both peristalsis and secretion in the large intestine, although van der Willigen (17), working with cats, failed to notice any abnormal secretion.

<sup>3</sup> Hurst (7), p. 349.

*Physiological Salt Solution.*—When the fecal reaction had remained normal for several days a pint of cold physiological salt solution was taken before breakfast. An hour later a stool was passed, the first part of which was normal in consistency but the last part of which was unusually soft. It was normal in reaction, having a pH of 7.3. 2 hours later a second movement occurred. The stool was liquid and somewhat more alkaline than the former one although its pH of 7.5 was still within the normal range. No other movements took place until the usual one on the following day which was normal in reaction.

The use of physiological salt solution as a laxative was suggested to the author some years ago. The explanation of its action was said to be the fact that, being isotonic with other body fluids it was not absorbed by the small intestine, which, of course, should not contain food, but was passed through to the large intestine where the increased bulk of its contents stimulated evacuation. Although this explanation may be open to question the fact remains that the drinking of a pint or more of physiological salt solution when the stomach is empty generally causes the passage of a copious fluid stool within an hour. Apparently its action is different from that of the other substances used in the present series of experiments, all of which tended to produce acid stools.

*Castor Oil.*—The reaction having remained constant for several days, a dose of castor oil was taken at 9 p.m. October 30. 6 hours later a small liquid stool was passed which was somewhat more alkaline than usual, having a pH of 7.6. It presumably consisted of the fecal matter already accumulated in the colon. 3 hours later a second liquid stool was passed. The reaction was slightly acid, pH 6.8. At the usual time, some 2 hours later the bowels were evacuated again, the stool being of a pasty consistency and having a normal reaction. At 2 p.m., however, an acid fluid stool was passed having a pH of 6.4. The following morning conditions were again normal.

From this point on the subject suffered from a protracted siege of digestive trouble which is distinctly reflected in the fecal reaction. The first symptoms immediately following the return to normal were those of acute diarrhea. The fecal reaction accordingly dropped and remained acid for 2 days (November 2 and 3). After a couple of normal days it again dropped with a mild initial

diarrhea and remained at about the neutral point for several days during which time the subject suffered from headache and general depression. This period finally culminated in one of constipation (November 12 to 14) during which the reaction rose to 7.9. After several days of low values, accompanied by symptoms similar to those experienced before, the normal range was resumed and adhered to with one exception.

This deviation occurred on November 25 and is of interest in showing the possible change in reaction within a single stool. The first portion passed was normal in appearance and had a reaction of 7.6 which is slightly more alkaline than usual. The last portion was a fluid, diarrheal stool and had an acid reaction of 6.7.

*Aloes*.—After several days of normal reactions 4 gr. of powdered aloes were taken with the evening meal on December 1. 12 hours later a soft stool was passed, the reaction of which was nearly neutral. It was followed 2 hours later by a semifluid movement having a normal reaction (pH 7.3), but 5 hours afterwards a fluid stool was passed which had a decidedly acid reaction, pH 6.2. By the following day the reaction had returned to the neutral point and remained in the normal range for several days.

*Senna*.—10 gr. of powdered senna were taken with the evening meal December 8. The result was the passage of two soft stools at intervals of 14 and 19 hours afterwards. These both tended towards low reactions although the second and most acid one attained a pH of only 6.7. The return to normal followed during the next 24 hours.

*Sulfur*.—5 gm. of powdered sulfur were taken with the evening meal December 16. No laxative action was apparent and there was no unusual change in the fecal reaction. The fecal material was somewhat softer than usual but the stools were well formed. There was a noticeable odor of sulfide.

It is apparent from these results that laxatives generally cause the lowering of the pH of the feces. All of those tested except physiological salt solution and sulfur (which latter produced no apparent laxative effect) resulted in a distinct increase in acidity. This was especially marked in the case of magnesium oxide whose alkaline properties did not come into evidence until after the cessation of its laxative action.

The result with physiological salt solution was the outstanding exception. Its laxative effect was apparently as thorough as that of magnesium oxide or castor oil, the stool being large and fluid, but the reaction was normal.

Diarrhea is accompanied by acidity and constipation by alkalinity of the excreta.

*The Influence of Biological Factors on the Fecal Reaction.*

As was stated above the bacterial flora of the intestine has been held chiefly responsible for changes in the fecal reaction, an abnormal acidity being due to the activities of acid-forming bacteria while an abnormal alkalinity has been attributed to the excessive development of putrefactive organisms. In view of the tremendous numbers of bacteria known to be present in the intestine this statement appears to be entirely warranted, but on account of the lack of any experimental evidence it must nevertheless be classed as an assumption. With the exception of Rettger's work the author has been able to find no record of any attempt to study the actual relations between the biological activities in the intestine and the fecal reaction, although a large amount of work has been done by bacteriologists on the intestinal flora and the conditions controlling it.

In the present study the results of Rettger and his associates have been utilized. They showed that the drinking of a quart of *Bacillus acidophilus* milk or the ingestion of large amounts of lactose will convert the usual mixed flora of the intestine into one which is almost a pure culture of *Bacillus acidophilus* in about 8 days or less.

*Buffer Action of Feces.*—In considering the possible change in reaction of a nutrient medium which growing organisms can produce, two factors must be considered, *i.e.* the fitness of the medium for the development of the organisms themselves and the readiness with which the reaction of the medium can be altered, in other words, its buffer power. There is no question about the suitability of the intestinal contents for the development of bacteria. Their ability then to change the reaction of the intestinal contents will depend upon the buffer power of this material, if we assume for the moment that no other factors enter into the case. Two

samples of fecal material were made into thin pastes containing, respectively, 3.7 and 3.9 per cent of solid matter. They together with a sample of plain broth, were then titrated with 0.1 N lactic acid and 0.1 N NaOH, 3 cc. samples being used. The results were plotted and are shown in Fig. 2. It is apparent that these suspensions which probably approximate in consistency those enter-

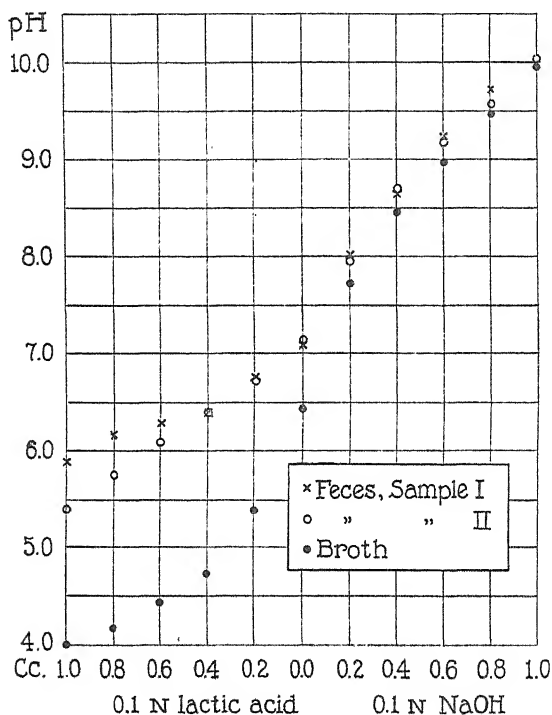


FIG. 2. Titration curves for feces and broth.

ing the cecum, are much more resistant to change of pH by the action of acid than is ordinary broth, the latter requiring only about  $\frac{1}{2}$  cc. of the acid to increase its acidity practically to the limit of growth of most organisms, whereas 1 cc. of the acid still left the reaction of the fecal suspensions well above this limit. Raising the pH above the neutral point takes place with equal ease in both media.

*Effect of Bacteria on the Reaction of Fecal Suspensions in Vitro.*—A sample of feces was next macerated with physiological salt solution in a mortar and the resulting suspension passed through a sieve to remove coarse debris. As finally used it contained 9.5 per cent of solid matter. 5 cc. portions were transferred to sterile test-tubes, some of which contained 0.1 gm. of lactose. Half were then autoclaved and each was inoculated with 1 cc. of a suspension (turbidity 4 on McFarland's scale (11) corresponding to about two billion bacteria) of one of the organisms as indicated. Table IV gives the reactions after 24 hours incubation at 37°.

TABLE IV.

Contents of tube.	Organism.	Reaction.	
		Unsterilized.	Sterilized.
		<i>pH</i>	<i>pH</i>
Feces (original material).		7.3	
"	None.	7.1	7.4
" + lactose.	"	5.0	6.8
"	<i>B. bulgaricus</i> .	6.7	7.3
" + lactose.	" "	5.0	5.6
"	" <i>acidophilus</i> .	6.7	7.1
" + lactose.	" "	5.0	4.9
"	" <i>mesentericus vulgaris</i> .	6.8	6.8
"	" <i>proteus vulgaris</i> .	8.0	7.2

During incubation the uninoculated, unsterilized material changed its reaction only 0.2 pH. The result is in agreement with observations of Howe and Hawk and the author's own experiences; *viz.*, that the reaction of fecal suspensions does not change greatly on standing for some hours.

The corresponding tube to which lactose was added developed a marked acidity indicating the presence of acid-forming organisms whose growth, in the absence of other factors was stimulated by the increased food supply. All of the lactose-containing tubes developed an acidity almost equal to the tolerance of the organisms, except the sterilized control tube which showed only a slight decrease in pH probably due to the splitting of the lactose during sterilization.

Evidently the presence of lactose is the determining factor in the development of fecal acidity by acid-forming organisms *in vitro*. Were it possible to carry the analogy of the test-tube to the intestine it would seem that a heavy lactose diet which causes the intestinal flora to become almost a pure culture of *Bacillus acidophilus* and which also causes the appearance of lactose (presumably) in the feces should produce some increase in the fecal acidity.

Rettger and Cheplin<sup>1</sup> determined the pH of the feces of subjects before and after the conversion of the mixed flora into one predominant with acid producers. They found that after the change in flora the reactions were still within the normal range; *i.e.*, the changes were no greater than the differences between individual subjects. Their method of procedure may be questioned, however. As was shown in a preceding section of this paper the fecal reaction usually lies within the limits pH 7.0 to 7.5, but values are frequently noted which lie without this range. In determining the range on a number of subjects on any 1 day the usual range may be considerably extended by the chance occurrence of one of several causes. An individual's normal reaction might be considerably lowered and still lie within such a range. For the same reason a single determination can hardly be relied upon to give a true picture of a subject's condition. When the reaction is followed from day to day, however, any protracted change can be detected by the lowering of the general level of the curve.

*Effect of Drinking Bacillus bulgaricus and Bacillus acidophilus Milk and of Lactose Feeding upon the Fecal Reaction.*—For this work each subject drank daily between 9 p.m. and 5 p.m. a quart of whole milk which had been heavily inoculated with the organisms and incubated for 12 to 18 hours at 37°.

Instead of taking mixed samples of the whole stools, portions from opposite ends were taken and tested separately. This was done because from the results the probable reaction of the mixed stool could be obtained as the average and also because it was found that certain relationships not apparent from figures with the mixed samples became plain when the method of sampling ends was employed.

As was pointed out above and shown in Table I the intestinal contents normally increase slightly in alkalinity during their

passage through the terminal part of the colon. It might be predicted that under the influence of a flora predominantly aciduric this condition would be reversed and the reaction become more acid. This appears to be what actually does take place in some

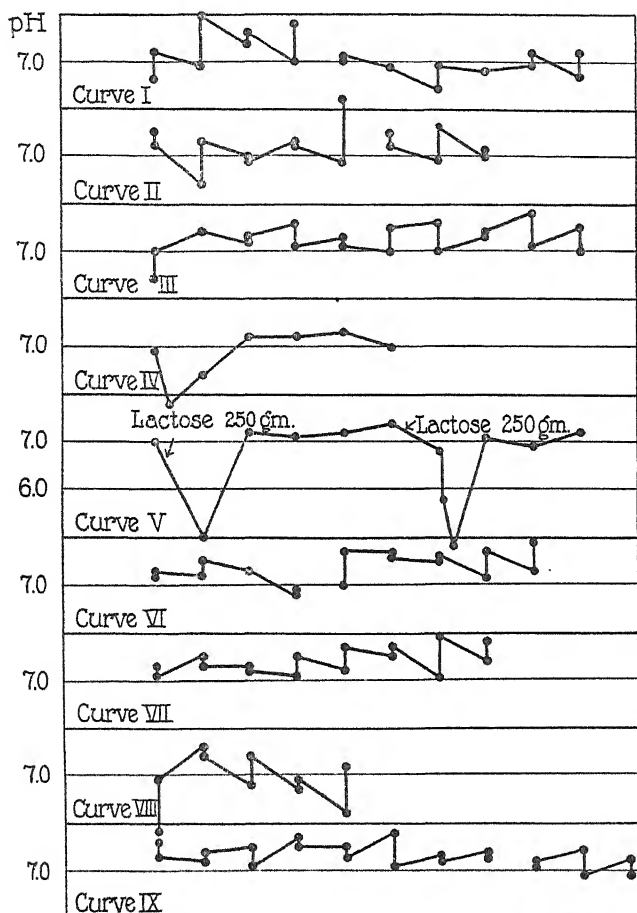


FIG. 3. Effect of bacteria and lactose on the fecal reaction.

cases and while, as Rettger and Cheplin claimed, the reaction of the material does not fall below the normal, due probably to a compensating physiological adjustment, a lack of food, or both, which tends to keep it near neutrality, the tendency towards acid formation is evidenced by the change in the type of curve.



In Fig. 3 are shown graphically the results of these experiments. In this figure the slanting parts of the curves represent the change in reaction within the stool itself while the vertical portions show the change in reaction of material in passing through the last part of the colon. Curve I shows a normal period. Curve II shows the reactions during the period of drinking a quart of *Bacillus bulgaricus* milk daily. In this case, however, the type of curve did not change. There are two exceptional values, but such occur occasionally even under ordinary circumstances and probably indicate a transient disturbance which normally is promptly corrected as it was in the present instance. This result substantiates in a chemical way Rettger's results. He found it impossible to implant *Bacillus bulgaricus* in the human intestine.

With *Bacillus acidophilus* milk the picture is quite different, the type of curve (Curve III) being completely changed and remaining so for some time after the cessation of treatment.

Curves IV and V show the effect of lactose feeding upon the fecal reaction as determined by mixed samples from the whole stools. Rettger observed an increased acidity with one subject which he attributed to some intestinal irritation. The curves in Fig. 3 show that this is probably correct. Curve IV, which represents results with Subject B, shows a pronounced drop in the fecal reaction with accompanying diarrhea which occurred at the start of the treatment. This effect was not noticed later when the work for Curves VI and VII was being done with the same subject so that it is not necessarily a constant one with any one person. With Subject A, however, it always took place whenever lactose was fed. He seemed to be peculiarly sensitive to lactose feeding, possibly because his usual diet was almost milk-free. The addition of a quart of whole milk or of a few grams of lactose daily to his diet was always accompanied by a drop in the curve though the values were never much below the neutral point. But when the attempt was made to give him 200 gm. of lactose per day the resulting diarrhea was so severe that the experiment had to be stopped. The fecal reaction was in this case markedly lowered. Curve V shows the result of these trials. Possibly infants may exhibit the same sensitiveness to this sugar, which would explain the reported acidity of the feces of infants while on a diet of mother's milk, which is richer in lactose than is cow's milk.

Curves VI to IX show the results of lactose feeding on Subjects B, C, and D, the ends of the stools being sampled. Two tests were made with Subject B with about a 2 week interval between them. Both curves were of the normal type. The presence of reducing substances in the feces was never demonstrated with certainty nor was there any unusual increase in acidity after the samples had been allowed to stand for 24 hours although during the last few days of the second experiment the amount of lactose taken was increased to about 400 gm. per day. Similar results were obtained with Subject C although Benedict's reagent showed the presence of reducing substances and the reaction decreased by from 0.5 to 0.7 pH on standing 24 hours. The reaction never dropped as low as when lactose was added directly to the fecal material as in the experiments described above.

With Subject D reducing substances were always present and the acidity was increased on standing to about the same extent as did the material from Subject C. The type of curve, however, changed to the form obtained when acidophilic milk was used.

Unfortunately, bacteriological examinations could not be made in connection with the chemical work, hence no positive statement can be made regarding the relationships between the above results and the changes in the bacterial flora. The true significance of the two forms of curves must therefore be uncertain. It may be that with Subjects B and C the flora remained unchanged, as Rettger found to be the case in one instance.<sup>4</sup> On the other hand, this feature may depend upon personal idiosyncrasies quite independent of the intestinal flora. The more probable explanation is that in some cases the increased activity of the acid-producing flora is counterbalanced by the increased activity of the compensatory mechanism of the intestine. It appears to be a safe assumption, based on Rettger's results, that the intestinal flora was converted in all cases.

It is very evident, however, that in no case was there a noticeable protracted increase in the acidity of the feces. In ordinary fecal material which has reached the colon such an increase is apparently prevented by a lack of available food even with a bacterial flora which is predominantly acidophilic, since the reaction fails to show a marked change on incubation after receiving a heavy inocu-

<sup>4</sup> Rettger and Cheplin (14), p. 70.

lation of *Bacillus acidophilus*. But the addition of lactose to such material either with or without inoculation is accompanied by the development of a degree of acidity practically equal to the limit of endurance of the organism. Hence the conclusion appears justified that one limiting factor in the development of acidity in ordinary fecal material after it reaches the colon is the supply of appropriate food. But when by heavy lactose feeding, suitable nutrient material does find its way into the lower intestine the regulatory mechanism of the bowel still prevents the attainment of a degree of acidity which is reached *in vitro*. There are then these two obstacles to the production of acidity in feces by biological means. They make untenable the assumption that the production of acid stools is ordinarily effected by bacteria.

The data regarding the factors instrumental in the production of fecal alkalinity are not so clarifying. The retention of feces

TABLE V.  
*Ammonia Content of Feces.*

	Sample I.		Sample II.	
	a	b	a	b
pH.....	7.9	6.6	8.0	7.4
NH <sub>3</sub> parts per million.....	468	884	251	503

in the colon is generally accompanied by an increase in the alkalinity. The biological explanation for this is that it is due to the production of ammonia by putrefactive organisms. This result does not appear to be a common one *in vitro* as it was observed in the present work only in one case; *i.e.*, one tube of material inoculated with *Bacillus proteus*. This result is doubtful since the same material which had been sterilized previous to inoculation showed no such behavior. Throughout the present work there was always production of acidity in material upon standing rather than an increase in alkalinity. This, together with the apparent tendency of the bowel to secrete alkali, makes the physiological explanation appear the more plausible. Additional support is given this contention by the following experiment.

The reaction of the samples from the ends of two stools was determined, the material made acid with acetic acid and then

dried on the steam bath. The dried material was then weighed into Kjeldahl flasks, water and magnesium oxide were added, and the ammonia was boiled off into 0.02 N acid. The results appear in Table V.

It is, of course, necessary for additional work to be done before definite conclusions can be drawn regarding this point. At the present time it can only be said that there is no experimental evidence that the alkalinity of feces is due to bacterial rather than physiological causes.

#### SUMMARY.

The normal fecal reaction of apparently healthy men on mixed diets lies between pH 7.0 and 7.5 though temporary variations beyond this range may occur without giving rise to any unusual sensations on the part of the subject.

The effects of some common laxatives on the fecal reaction were studied. The usual result is the production of an acid stool. The administration of the alkali, magnesium oxide, does not differ from the others in this respect but the fecal material passed after the cessation of the laxative action is unusually alkaline.

The physiological factor is the predominant one in influencing fecal reaction, no result being noticeable from the introduction of acidophilic bacteria into the intestine. The intestine apparently exerts a regulatory influence which prevents the development of acidity by microorganisms. There is evidence that alkalinity in feces is also due to physiological rather than biological causes.

In conclusion the author desires to express his thanks to Dr. W. L. Chandler for suggestions regarding this work and to Mr. R. L. Tweed for supplying bacterial cultures and assistance in certain experiments.

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## STUDIES OF AUTOLYSIS.

### VIII. THE NATURE OF AUTOLYTIC ENZYMES.

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Dernby (1, 2) has recently advanced the theory that the enzymes concerned in autolysis of tissues are mixtures of pepsin, erepsin, and trypsin, and has presented evidence to support this view from a study of protein digests made up with mixtures of these enzymes. His artificial mixtures show optimum amino-acid production in a medium which is somewhat acid (pH 5 to 6) just as is the case in autolyzing gland tissues and at this H ion concentration all three of these enzymes can act.

While this conception of the autolytic mechanism appears to simplify and explain many of the phenomena of autolysis, it is not in harmony with all the facts, nor are the experiments presented by Dernby of such a crucial character as to be entirely convincing. As a matter of fact there is much evidence in Dernby's own data which must cast doubt on the validity of this hypothesis. It must be questioned whether applying the names of such well known and definitely characterized proteolytic complexes as pepsin and trypsin to the enzymes of tissues does not in the end complicate rather than clarify the situation. Since these names connote very definite properties, developed by decades of research, it must result in a certain confusion of ideas to apply them to other protease groups, unless these latter are found to have closely similar properties.

Before accepting Dernby's classification of the enzymes concerned in tissue breakdown as closely related to pepsin, erepsin, and trypsin, it should be possible to subject tissues to such crucial conditions as to develop conclusively the properties of these enzymes if they are actually present. Unless there is positive evidence

of the very close similarity of properties, these historic names should not be applied. In the case of erepsin, or the ereptases, there is evidently much more latitude allowable than in the case of pepsin and trypsin. The ereptases—enzymes which do not digest the ordinary native proteins, but do hydrolyze their primary cleavage products—are widely distributed in animal tissues (3 to 6). There may indeed be ground for believing this type of enzyme in the tissues to be identical with the erepsin of the small intestine. It is not the purpose of this study to investigate properties of the ereptic group of proteases. It is our intention, however, to apply selected tests to tissues whose autolysis is best known, with a view to proving definitely the presence of pepsin and trypsin, or their absence. This may be done in two ways. One involves adding pepsin and trypsin to autolyzing material and determining in what ways this changes the digestive picture, the other involves subjecting the tissues to extreme conditions, under which pepsin or trypsin may still function, but which bring autolysis to a complete standstill.

At the outset it seems advisable to define our terms in accord with the widely accepted conceptions of pepsin and trypsin and to summarize concisely the outstanding features of organ autolysis pertinent to this investigation.

*Trypsin* is that enzyme complex first found secreted by the pancreas and extractable from the gland itself (7 to 10). It is characterized by the fact that it reacts in acid, neutral, and alkaline media, or between a pH  $9\pm$  to about  $4.5+$ . Its optimum is in the neighborhood of 7.8. It digests many native proteins, as of the tissues,<sup>1</sup>

<sup>1</sup> On pages 187 and 188 Dernby (2) makes the following assumption which is misleading: "Most native animal proteins, are attacked by pepsin but not by trypsin, the latter enzyme acting only on the products of peptic digestion." On page 210 appears the statement "In alkaline solutions . . . trypsin can digest only the peptones which are present from the beginning," and later "Without the pre-treatment in acid most of the material would have remained in the form of native protein indigestible by trypsin." This conception of tryptic activity is certainly not the usual one, and does not coincide with the widely accepted data on it (10). We have many experiments which indicate that liver and kidney at least are very completely and rapidly hydrolyzed if trypsin is added, and that without any chance for preliminary peptic cleavage. Dernby's own experiments furnish evidence of this also; for example Table XXVIII shows the pancreas autolyzing best at an initial pH of 7.9, which makes preliminary peptic digestion impossible.

quite rapidly, although the skeletal or connective tissue group is resistant to it. Hydrolysis proceeds to the amino-acid stage, with the cleavage of the proteose and peptone fragments so rapid, that these are never found in large amounts in a typical tryptic digestion. While preliminary peptic digestion of native proteins unquestionably facilitates subsequent tryptic digestion, it is by no means essential where tissues are being used as substrata, as will appear in the experiments below. Trypsin can hydrolyze the liver and kidney proteins at such low H ion levels that peptic cleavage is entirely ruled out. Whether the action of the pancreatic extract is due to trypsin alone, or to a mixture of trypsin and erepsin is of no significance in this connection since erepsin is conceded present in both the pancreas and the other organs such as kidney and liver. Tryptic digestion may be most conveniently estimated by the amino-acid production.

*Pepsin* is the proteolytic enzyme characteristic of the gastric juice (11 to 16). It hydrolyzes practically all the common native proteins, with the exception of keratin, and in a medium ranging from a H ion of pH  $1.5 \pm$  to 7.0-. By the action of acid-pepsin the native proteins are rapidly broken up into the larger polypeptides, constituting the proteose and peptone groups. Amino-acids are split off during peptic digestion, but at an extremely slow rate (cf. Table IV). Peptic digestion cannot be estimated satisfactorily therefore by amino-acids, but by some measure of initial cleavage, such as non-coagulable nitrogen, non-precipitable nitrogen, or by the colorimetric measurement of tyrosine-containing fragments. This latter method we have used as our criterion of initial cleavage.

*Autolysis* of liver and kidney is characterized by the following outstanding features (17). At a pH of 7 to 8 autolysis is at a standstill, at higher H ion levels it proceeds more and more rapidly and in rough proportionality to the increased acidity to an optimum, at pH 4.0 to 4.5 (if HCl is added). Strictly speaking an extremely slow hydrolysis can be recognized at pH 7 to 8, but it is so small as to be of no significance in this discussion. At optimum acidity all of the gland but a small residue, which consists chiefly of connective tissue, pigment, and nucleins digests to amino-acids. The optimum acidity is approximated in our experiments by adding 25 to 40 cc. of 0.2 N HCl to 50 gm. of tissue, made up to a final



volume of 250 cc. Where more acid is added autolysis is diminished, and at a pH 3 to 2.5, corresponding to 75 to 100 cc. of 0.2 N HCl in the digest, autolysis is practically at a standstill. What little digestion occurs at this level may very well be due to acid alone and in any event is too small to be of significance here.

With the above summaries much of Dernby's experimental data is in complete accord. For example his artificial mixtures of pepsin and trypsin digest gelatin very well at the highest acidity reported (pH 2) and the curves indicate that at a pH of 1+ peptic digestion would have gone on. The same is true when this artificial mixture of enzymes acts on coagulated liver. There is 50 per cent of the optimum activity at a pH 2, where trypsin is largely inhibited. But when fresh liver suspension is used<sup>2</sup> there is no evidence of gelatin digesting at pH 2.6, though it does go well at pH 3.8. In other words digestion of gelatin by liver, is completely inhibited by an acidity where pepsin is at its optimum.

There is much further evidence presented in Dernby's excellent paper which points to the very real differences between pepsin, trypsin, and the autolytic enzymes of such tissues as the liver. The data which follow bring out these differences so clearly, that we are convinced that the classic names trypsin and pepsin cannot advantageously be applied to the enzymes of such tissues as liver or kidney.

#### EXPERIMENTAL PART.

The procedure followed is that which we have used in previous investigations (18). In it we measure the final stage of proteolysis by titrating amino-acids.

To study the earlier steps of cleavage we have applied the colorimetric method of Folin and Denis (19) to the same trichloroacetic acid filtrates, used for amino-acid estimations. 5 cc. of these filtrates are treated with 5 cc. of the reagent and after standing 5 minutes, 20 cc. saturated  $\text{Na}_2\text{CO}_3$  solution are added, and the mixtures made up to 50 cc. These are then compared with standards, containing 0.5, 1.0, and 1.5 mg. of tyrosine, respectively, using that standard which appears to be nearest the concentration of tyrosine in the unknown. The materials which give the color are assumed to be polypeptides containing tyrosine together with some free tyrosine.

<sup>2</sup> Dernby (2), p. 193.

*Effect of Trypsin.*—The effect of trypsin on gland tissue may be illustrated by the autolysis of pancreas itself at various H ion levels. It is seen to digest best at a H ion level of pH 8+, and is very active even at pH 9.3 (initial).

If trypsin is added to some other tissue such as kidney or liver its presence alters the picture of autolysis in a characteristic way.

The data presented below, typical digests confirmed by many similar ones in our study of this problem, show quite clearly that when trypsin is known to be present the kidney suspensions digest best in the low H ion levels produced by added alkali. With increasing acidity the effect of trypsin grows less, and in the range

TABLE I.  
*Pig's Pancreas.*

No.	Condition.	pH	0.20 N amino-acid.					Net gain.
			Days.					
			0	1	3	7	20	
			cc.	cc.	cc	cc.	cc.	cc.
I	Control.....	6.30	0.35	3.30	4.10	4.40	5.00	4.65
II	“ + 25 cc. HCl.....	4.75	0.35	2.20	3.85	4.30	5.00	4.65
III	“ + 50 “ “ .....	4.00	0.35	1.15	1.80	2.60	3.05	2.70
IV	“ + 100 “ “ .....	2.60	0.35	0.40	0.40	0.50	0.50	0.15
V	“ + 200 “ “ .....	1.20	0.35	0.40	0.40	0.50	0.50	0.15
VI	“ + 12.5 “ NaOH....	7.02	0.35	3.85	4.40	4.40	5.05	4.70
VII	“ + 25 “ “ .....	7.78	0.35	4.25	4.75	4.60	5.40	5.05
VIII	“ + 50 “ “ .....	8.40	0.35	3.95	4.40	4.50	5.70	5.35
IX	“ + 100 “ “ .....	9.36	0.35	3.15	3.65	4.00	4.90	4.55

of acid giving optimum autolysis, the effect of trypsin is quite small. The mixture containing trypsin digests best in an alkaline medium—pH 8—where autolysis alone is practically at a standstill. This is clear evidence that trypsin does catalyze the initial cleavage of these native tissue proteins without preliminary acid cleavage. Furthermore the actual amino-acid level reached in the time of the digest is seen to be higher than when autolysis is allowed to proceed under its optimum conditions; *i.e.*, with 25 to 50 cc. of 0.2 N HCl present. This means evidently that more proteins are hydrolyzed by trypsin than by the autolytic enzymes alone, even under the best environment.

TABLE II.  
*Beef Kidney.*

No.	Condition.	pH	0.20 N amino-acid.				Net gain.	Gain due to trypsin.
			Days.					
			0	1	3	8		
			cc.	cc.	cc.	cc.	cc.	
I	Control.....	6.3	0.50	0.60	0.70	0.75	0.25	
II	“ + trypsin.....		0.55	2.70	3.60	3.60	3.10	2.85
III	“ + 10 cc. HCl.....	5.5	0.50	1.00	1.40	1.45	0.95	
IV	“ + 10 “ “ + trypsin.....		0.55	2.40	3.60	3.75	3.20	2.25
V	“ + 25 “ “ .....	4.7	0.50	2.00	2.90	3.15	2.65	
VI	“ + 25 “ “ + trypsin.....		0.55	2.10	3.10	3.55	3.05	0.40
VII	“ + 50 “ “ .....	4.0	0.50	1.65	2.45	2.75	2.25	
VIII	“ + 50 “ “ + trypsin.....		0.55	2.00	2.70	3.20	2.70	0.45
IX	“ + 25 “ NaOH .....	7.8	0.50	0.60	0.65	0.65	0.15	
X	“ + 25 “ “ + trypsin.....		0.55	3.30	3.90	3.90	3.35	3.20

TABLE III.  
*Beef Kidney.*

No.	Cndition.	Tyrosine in 5 cc. filtrate.				Net gain.	Gain due to trypsin.
		Days.					
		0	1	3	8		
		mg.	mg.	mg.	mg.	mg.	mg.
I	Control.....	0.15	0.17	0.17	0.17	0.02	
II	" + trypsin.....	0.15	0.40	0.40	0.40	0.25	0.23
III	" + 10 cc. HCl .....	0.15	0.20	0.23	0.23	0.08	
IV	" + 10 " " + trypsin...	0.15	0.34	0.40	0.40	0.25	0.17
V	" + 25 " " .....	0.15	0.32	0.37	0.37	0.22	
VI	" + 25 " " + trypsin...	0.15	0.34	0.40	0.40	0.25	0.03
VII	" + 50 " " .....	0.15	0.32	0.37	0.37	0.22	
VIII	" + 50 " " + trypsin...	0.15	0.34	0.40	0.40	0.25	0.03
IX	" + 25 " NaOH .....	0.15	0.15	0.15	0.15	0.00	
X	" + 25 " " + trypsin...	0.15	0.40	0.40	0.40	0.25	0.25

If the liberation of tyrosine be taken as a criterion of early cleavage, we again find a very marked contrast between the behavior of tissue alone and when supplemented by trypsin. The cleavage of the protein into fragments sufficiently small for the development of this color reaction takes place very rapidly when

trypsin is present, whether the mixture is acid, neutral, or alkaline. The less acid, however, the more rapidly is the tyrosine complex split off. In autolysis alone the liberation of tyrosine in alkaline mixture is so small as to be inappreciable.

*Summary.*—(a) With trypsin present the gland tissues studied—pancreas, kidney, and liver—behave alike. Initial cleavage measured by the tyrosine reaction goes on faster in alkaline than in acid mixtures. Under identical conditions, but without trypsin, the autolytic enzymes effect the least amount of initial cleavage, and in the case of beef kidney this may actually be zero. This is evidently not merely a quantitative difference, but a genuine difference in kind. (b) With trypsin present, amino-acids appear most rapidly in strongly alkaline mixtures, where autolytic digestion is practically at a standstill, when tested by the same criterion. Here again we have evidence of a difference in kind, rather than a difference in degree.

By both methods the unaided tissue fails to show evidence of trypsin under just those conditions where trypsin acts best. If trypsin is not to be found under its own optimum conditions, we are justified in concluding that it is not there. Although there are certain quantitative differences between autolysis of kidney and liver, the latter behaves so much like the former that we need not present the experimental figures to apply the same conclusions to it.

*Effect of Pepsin.*—It is distinctly more difficult to devise a series of experiments which shall serve to prove or disprove the presence of pepsin in a tissue. The very fact that autolysis proceeds better with increasing acidity makes the hypothesis that the enzyme complex facilitating autolysis is a pepsin-erepsin mixture all the more tenable. Furthermore, the inhibition of autolysis by still higher acidity is by no means a sharp or clean-cut phenomenon, and in some livers it is less sharp than in others.

If pepsin is allowed to act on coagulated liver, using the same amounts of tissue and the same final volume as in our standard autolysis technique, we have an approximate measure of the rate of amino-acid production which this enzyme alone may produce when added to uncoagulated liver, and we can get a fair indication of the optimum acidity. As is seen in Table IV, the production of amino-acids is very slow, as compared with autolysis, and it

increases with increasing acid up to a high H ion concentration (pH 2.0). There is a rough proportionality between the amount of acid added and the amino-acids produced.

If pepsin is added to a fresh tissue mixture, under varying pH, we find that it increases autolysis to some extent over the whole range, *but least at the optimum acidity for autolysis itself*. At this concentration of acid the primary fragmentation of the native tissue proteins goes on so fast that the addition of pepsin does not markedly alter the picture as a whole. If this initial fragmentation is due to pepsin, that enzyme must be there in abundance, and it should be easy to recognize it at the higher acidities where pepsin is known to act best.

TABLE IV.  
*Coagulated Liver and Pepsin.*

No.	Condition.	0.20 N amino-acid.			Net gain.	Gain due to HCl.
		Days.				
		0	3	13		
		cc.	cc.	cc.	cc.	cc.
I	Control (50 gm. coagulated liver, 10 cc. 5 per cent pepsin).....	0.25	0.30	0.35	0.10	
II	Control + 10 cc. HCl.....	0.25	0.30	0.35	0.10	0.0
III	" + 25 " " .....	0.25	0.35	0.40	0.15	0.05
IV	" + 50 " " .....	0.25	0.45	0.55	0.30	0.20
V	" + 75 " " .....	0.25	0.50	0.60	0.35	0.25
VI	" + 100 " " .....	0.25	0.55	0.75	0.50	0.40
VII	" + 125 " " .....	0.25	0.60	0.80	0.55	0.45

In Table V, the addition of 25 cc. of 0.2 N HCl gives the optimum autolysis. Pepsin scarcely increases the amino-acid titer at any stage of the 8 day autolyses. The addition of 50 cc. of 0.2 N HCl is sufficient to decrease autolysis while with pepsin added it is not decreased. Evidently here the change from 25 to 50 cc. of acid is sufficient to inhibit slightly the initial cleavage in autolysis alone. The subsequent digestion of primary cleavage products is not affected, since the addition of pepsin produces the same amino-acid titer as in the optimum autolysis.

In Table VI this interesting phenomenon is still more evident. Pepsin markedly increases amino-acid in the control, less at the optimum for autolysis, and more again when acid inhibition of

TABLE V.  
*Beef Kidney.*

No.	Condition.	0.20 N amino-acid.				Net gain.	Gain due to pepsin.
		Days.					
		0	1	3	8		
		cc.	cc.	cc.	cc.	cc. %	cc.
I	Control.....	0.50	0.60	0.70	0.75	0.25	
II	“ + pepsin.....	0.50	0.70	0.85	0.85	0.35	0.10
III	“ + 10 cc. HCl.....	0.50	1.00	1.40	1.45	0.95	
IV	“ + 10 “ “ + pepsin..	0.50	1.20	1.60	1.80	1.30	0.35
V	“ + 25 “ “ .....	0.50	2.00	2.90	3.15	2.65	
VI	“ + 25 “ “ + pepsin..	0.50	2.10	3.00	3.25	2.75	0.10
VII	“ + 50 “ “ .....	0.50	1.65	2.45	2.75	2.25	
VIII	“ + 50 “ “ + pepsin..	0.50	2.00	2.80	3.25	2.75	0.50

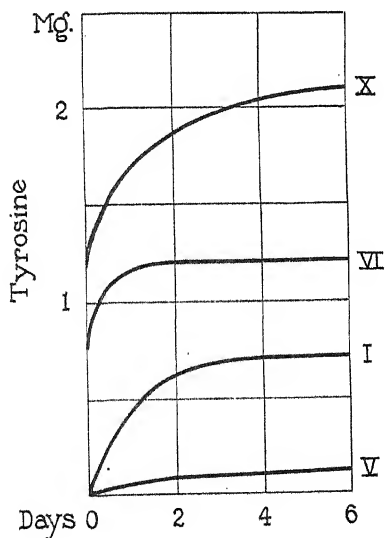


FIG. 1.

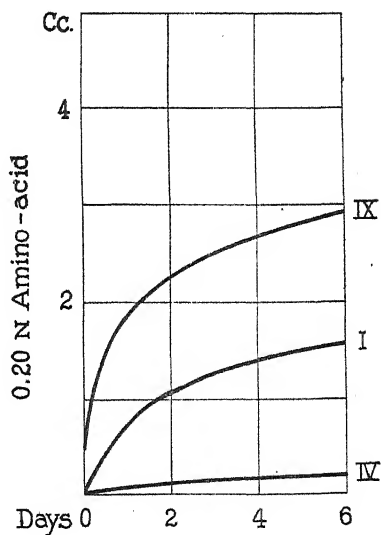


FIG. 2.

TABLE VI.

*Pig's Liver.*

No.	Condition.	pH	0.20 N amino-acid.						Net gain.	Gain due to acid.	Gain due to pepsin.
			Days.								
			0	1	3	6					
			cc.	cc.	cc.	cc.	cc.	cc.			
I	Control.....	6.30	0.30	1.10	1.40	1.85	1.55				
II	" + 25 cc. HCl.....	4.75	0.30	2.10	2.75	3.00	2.70	1.15			
III	" + 50 " ".....	4.00	0.30	2.15	2.70	2.70	2.40	0.95			
IV	" + 100 " ".....	2.60	0.30	0.40	0.50	0.45	0.15				
V	" + 200 " ".....	1.18	0.30	0.35	0.40	0.45	0.15				
VI	" + pepsin.....		0.60	2.90	3.10	3.60	3.00			1.45	
VII	" + " + 25 cc. HCl.....		0.60	3.30	3.10	4.10	3.50			0.80	
VIII	" + " + 50 " ".....		0.60	3.15	3.50	4.50	3.90			1.50	
IX	" + " + 100 " ".....		0.60	2.50	3.00	3.50	2.90			2.75	
X	" + " + 200 " ".....		0.60	1.75	1.55	1.85	1.25			1.10	
XI	" (coagulated).....		0.40	0.40	0.30		0.00			0.10	
XII	" + pepsin.....		0.60	0.80	0.70		0.10				
XIII	" + 50 cc. HCl.....		0.40	0.45	0.45		0.05	0.05			
XIV	" + 50 " " + pepsin.....		0.60	0.95	1.00		0.40			0.35	
XV	" + 100 " ".....		0.40	0.40	0.45		0.05	0.05			
XVI	" + 100 " " + pepsin.....		0.60	1.55	1.50		0.90			0.85	
XVII	" + 200 " ".....		0.40	0.45	0.50		0.10	0.10			
XVIII	" + 200 " " + pepsin.....		0.60	1.60	1.75		1.15			1.05	

autolysis begins to appear (*cf.* Nos. I to VIII in this series). When the acidity reaches the level represented by No. IV (pH 2.6), autolysis is completely stopped (*cf.* Figs. 1 and 2). But the action of pepsin in No. IX at this level is much better than at the level of Nos. III and VIII. This coincides with its action on the same liver when coagulated, as Nos. XV and XVI. At the same time, making all possible allowance for the amino-acids produced by pepsin alone (0.85 cc.), we find the further digestion of the primary cleavage products proceeding at a rapid rate. Thus in No. IX as in No. IV, autolytic primary cleavage is negligible. The total amino-acids, therefore, must result from pepsin and the erepsin of the tissue. Subtracting the amino-acids referable to pepsin, we get  $2.75 \text{ cc.} - 0.85 \text{ cc.} = 1.90 \text{ cc.}$  of amino-acids resulting from the ereptic disintegration of the products of peptic digestion. In Nos. V and X the ereptic enzymes are inhibited. The amino-acids found in No. X are produced by pepsin alone, and correspond closely with those produced in No. XVIII.

In Table VII the rate of initial cleavage is determined by the tyrosine reaction. The figures are not as accurate as they are in the amino-acid titrations, but they are sufficiently so to show that primary cleavage goes on when pepsin is present, in this series Nos. IX and X, at an acid level which completely inhibits cleavage by the autolytic enzymes themselves, Nos. IV and V. (The cleavage in Nos. IV and V corresponds fairly well with that produced by acid alone on coagulated liver proteins, *cf.* Nos. XV and XVII.) In short we find that primary cleavage by the proteases of the liver is completely inhibited at pH 2.6+, while with pepsin present, primary cleavage goes on best at pH 1.2+. The inhibition of autolysis as a whole at pH 2.6+ is due to inhibition of primary cleavage and is not due to inhibition of the erepsin effect.

In Fig. 3 the behavior of liver mixture to varying amounts of acid is shown, at the end of the 1st and 6th day. With pepsin present, initial cleavage increases with acidity throughout the entire range of the experiment (pH 6.2 to 1.2). With pepsin absent, the optimum, followed by complete inhibition, form the outstanding features.

In Fig. 4 is shown the effect of pepsin on the amino-acid production in autolyzing liver. The dotted line indicates amino-



TABLE VII.  
*Tyrosine from Pig's Liver.*

No.	Tyrosine in 5 cc. filtrate.				Net gain.	Gain due to pepsin.
	Days.					
	0	1	3	6		
	mg.	mg.	mg.	mg.	mg.	mg.
I	0.37	0.77	1.13	1.13	0.76	
II	0.37	1.43	2.13	2.13	1.76	
III	0.37	1.56	2.20	2.13	1.76	
IV	0.37	0.52	0.56	0.55	0.18	
V	0.37	0.45	0.50	0.50	0.13	
VI	0.41	1.90	1.90	1.81	1.40	0.64
VII	0.41	2.25	2.50	2.50	2.10	0.34
VIII	0.41	2.17	2.50	2.50	2.10	0.34
IX	0.41	2.31	2.25	2.50	2.10	1.92
X	0.41	2.75		2.75	2.34	2.21
XI	0.43	0.38	0.25	0.28	0.0	
XII	0.63	0.63	0.48	0.56	0.0	
XIII	0.43	0.43		0.58	0.15	
XIV	0.63	1.15	1.38	1.44	0.81	0.66
XV	0.43	0.50	0.50	0.48	0.05	
XVI	0.63	1.45	2.13	2.25	1.63	1.58
XVII	0.43	0.53	0.50	0.55	0.12	
XVIII	0.63	1.45	2.13	2.31	1.68	1.56

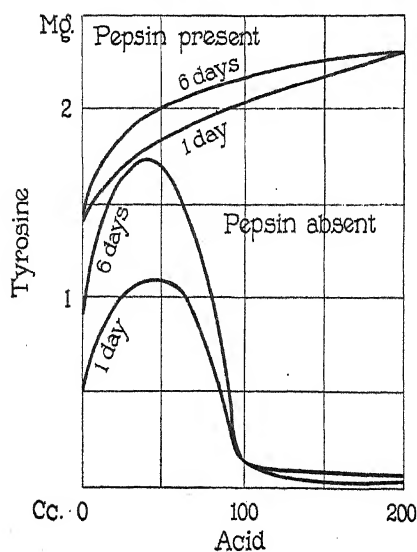


FIG. 3.

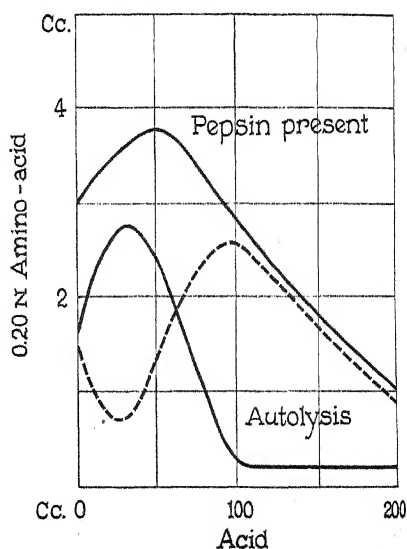


FIG. 4.

acids referable to the activity of pepsin in the mixture. The maximum effect appears where autolysis is minimal.

*Effect of Preliminary Acidification.*—If a tissue is first made acid, and later this acid exactly neutralized, the tissue has been altered so that autolysis will now proceed in a neutral, acid, or alkaline medium. This phenomenon was described by Dernby and we have repeatedly confirmed the observations. The initial cleavage is quite rapid as compared with autolysis as a whole, but is not complete in 24 hours, as is seen in Table VIII.  $\frac{1}{2}$  hour contact does not measurably alter autolysis;  $3\frac{1}{2}$  hours give a small increase of amino-acids; longer contact gives proportionately larger final digestion.

In Table IX this is shown in another way. After 24 hours the acid added to No. VI is exactly neutralized. At the end of 2 days it and the acid control are just alike, but thereafter the neutralized digest falls behind. The primary cleavage was sufficient to supply an excess of substratum for the ereptic enzymes for 24 hours, after which the diminishing mass of substratum is apparent. Where more than the requisite amount of alkali to neutralize HCl is added—as in Nos. IV and V—the total autolysis is still more definitely inhibited, when compared with the digest which remains acid throughout. This suggests that in the acid mixture there is an advantage in more than the initial cleavage of tissue proteins. This is a point which needs further study.

*Summary.*—The primary cleavage of tissue proteins in autolysis is effected by an enzyme acting only in acid media. To this extent it resembles pepsin in its properties.

This enzyme, however, is destroyed at pH 2.6+ at which pepsin is very active. The optimum for pepsin is about pH  $1.5\pm$  and it is not destroyed till a much higher acidity is reached. The optimum for autolysis is about pH 4.5. Inasmuch as the conversion of polypeptides to amino-acids goes on very rapidly at this H ion level and considerably higher, it is fair to assume that this is approximately the optimum pH for the enzyme which effects the initial cleavage of the tissue proteins. This is corroborated by the fact that the liberation of reactive tyrosine peptides goes on best between pH 4.7 and 4.0. When pepsin is present tyrosine production goes on best at the high acidity of pH 1.2.

TABLE VIII.

*Beef Kidney.\**

No.	Condition.	Time of contact.	0.20 N amino-acid.												Net gain.
			Hours.						Days.						
			0	$\frac{1}{2}$	$3\frac{1}{2}$	$10\frac{1}{2}$	22	2	5	13					
			cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.					
I	Control.....		0.40			0.65	0.65	0.80	1.00	1.00	1.00	0.60			
II	" - 25 cc. HCl.....	$\frac{1}{2}$	0.40	0.55	0.60	1.25	1.85	2.25	2.80	3.10	3.10	2.70			
III	" - 25 " ".....		0.40	0.55			0.70	0.80	1.00	1.00	1.00	0.60			
IV	" - 25 " ".....	$3\frac{1}{2}$	0.40		0.65		0.95	1.20	1.20	1.25	1.25	0.85			
V	" - 25 " ".....	$10\frac{1}{2}$	0.40			1.30	1.75	1.95	2.15	2.15	2.15	1.75			
VI	" - 25 " ".....	22	0.40				1.85	2.25	2.45	2.45	2.75	2.35			

\* 25 cc. of NaOH were added at the end of the period indicated.

TABLE IX.

*Beef Liver.\**

No.	Condition.	0.20 N amino-acid.						Net gain.	Gain after 1st day.
		Days.							
		0	1	2	5	11	cc.		
	Control	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
I	"	0.40	1.20	1.40	1.60	1.65	1.25		
II	" + 25 cc. HCl	0.40	1.65	2.40	3.10	3.35	2.95		1.70
III	" + 25 " NaOH	0.40	0.65	0.70	0.85	0.85	0.45		
IV	" + 25 " HCl + 50 cc. NaOH	0.40	1.65	2.00	2.40	2.50	2.10		
V	" + 25 " " + 37½ "	0.40	1.65	2.15	2.50	2.80	2.40		0.85
VI	" + 25 " " + 25 "	0.40	1.65	2.40	2.65	2.90	2.50		1.15
VII	" + 25 " " + 20 "	0.40	1.65	2.30	2.90	3.25	2.85		1.25
VIII	" + 25 " " + 15 "	0.40	1.65	2.30	2.80	3.25	2.85		1.60
IX	" + 25 " " + 10 "	0.40	1.65	2.50	3.10	3.45	3.05		1.60

\* Alkali was added to Nos. 4 to 9 after 24 hours contact with acid.

These differences in properties are sufficiently striking to make it evident that the name pepsin cannot appropriately be applied to this enzyme.<sup>3</sup>

As a provisional extension of the conception of the autolytic mechanism which we put forward in 1916 (18), and in view of the more recently developed data the following hypothesis is presented as a brief statement of the autolytic mechanism: (a) In the normal living cell, with an average pH of 7.4 the tissue proteins are present in the form of base-protein combinations—Na, K, and Ca salts of the proteins (20). These protein salts are not substrata for the autolytic enzymes of the cell, and remain undigested so long as the normal H ion level prevails. (b) When acid develops it combines with the bases dissociated from the proteins and the latter become acid-protein salts (or possibly the free proteins). (c) These acid-protein salts are available material for primary cleavage, catalyzed by an enzyme which we may designate as the primary protease of the tissue (the "Beta protease" of Hedin (21), and the "endotryptase" of Hahn (22)). This cleavage results in smaller polypeptide fragments of the general character of proteoses and peptones. (d) The fragments produced in this initial primary cleavage, which can only take place as acid is developed within the cell, are then rapidly converted into amino-acids by the active ereptase present in the tissue. This step in the process proceeds in either acid, neutral, or alkaline media.

So long as the base-protein salts remain there is no possibility of autolysis, since there is no substratum for the primary protease to act upon. The instant acidotic changes occur in the cell, with the conversion of base-proteins into acid-proteins, the latter are removed through the autolytic machinery. The tissue proteins are thus reservoirs of bases which may be called out for the buffering of an acidotic shift in the acid-base balance, just as happens

<sup>3</sup> It should be noted that the  $\beta$ -protease of the spleen, as described by Hedin, is an enzyme acting only in acid media producing amino-acids, and far less sensitive to  $\text{Na}_2\text{CO}_3$  than pepsin.  $\beta$ -protease also acts better in dilute acetic acid (0.2 per cent) than in the same concentration of HCl. It is probable that Hedin actually had a mixture of two enzymes, but the differences in properties were sufficiently striking so that he concluded this enzyme could not be pepsin. He also concluded that  $\alpha$ -protease, reacting best in alkaline medium, could not properly be classed as trypsin.

in the blood. But in the cell this leads to atrophic changes tending to reduce its mass and its metabolism to a point where it is again maintained in acid-base equilibrium by the blood and lymph supply. When this equilibrium is attained the autolytic shrinkage of cell or tissue automatically ceases.

#### CONCLUSIONS.

1. Applying a crucial type of test for trypsin to liver or kidney fails to disclose evidence of an enzyme with the characteristic behavior of pancreatic trypsin.

2. Similarly crucial tests fail to develop evidence of an enzyme which behaves sufficiently like gastric pepsin to warrant including it under that name.

3. There is evidence of an ereptic type of enzyme, which digests the primary cleavage products of proteins to amino-acids, but which does not digest the native tissue proteins. This enzyme complex is active between the H ion levels of pH 8 to 3-. It is completely inactive at pH 1+. It is present in the tissues studied in abundance and is apparently not a limiting factor in the rate of autolysis under conditions met with in the body.

4. There is evidence of an enzyme complex which digests the acid-salts of the tissue proteins between pH 7 and 3. It is completely inhibited at a H ion level of pH 2.6 while pepsin remains active in a H ion concentration of pH 1±. We have designated it here the *primary protease* of the tissue, since it catalyzes the initial cleavage. The action of this enzyme constitutes the limiting factor in the autolytic machinery, and its activity is in turn conditioned by the amount of acid produced.

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## ON A POSSIBLE ASYMMETRY OF ALIPHATIC DIAZO COMPOUNDS. II.

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It has long been known that the esters of optically active amino-acids on treatment with nitrous acid give rise to esters of the corresponding hydroxy-acids. Likewise esters or inner esters (lactones) of amino sugar acids give rise not to a mixture of the esters of two epimeric hydroxy-acids, but to one single substance. It has also been known for some time that on mild treatment of the esters of amino-acids with nitrous acid, diazo derivatives and not hydroxy-acid esters are formed.

Levene and La Forge<sup>1</sup> in 1915 made the observation that the esters of benzilidene ethyl chitosaminates on mild treatment with nitrous acid gave rise to a diazo derivative. This observation was an impetus to a scrutiny into a possible connection between the formation of the diazo derivative and the hydroxy-acid. It seemed possible to regard the diazo derivative as an intermediary phase in the transformation of the esters of amino-acids into the esters of hydroxy-acids. If this were proven it would necessitate the assumption of the existence of enantiomorphous aliphatic diazo esters.

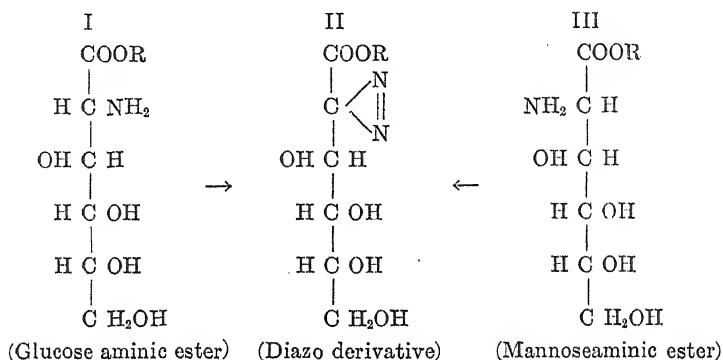
This part of the problem required experimental verification. Work was in progress in this laboratory for several years on substances of the two mentioned groups. In 1920 Marvel and Noyes<sup>2</sup> criticized the deductions which Levene made from his observations on the behavior of hexosaminic acids. According to Marvel and Noyes the formation of one hydroxy-acid (and not a pair of epimeric acids) may be readily explained by the presence of four asymmetric carbon atoms in the molecule of

<sup>1</sup> Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 345.

<sup>2</sup> Marvel, C. S., and Noyes, W. A., *J. Am. Chem. Soc.*, 1920, xlii, 2259.



these substances and on the assumption that the configuration of carbon atoms 3, 4, and 5 control the configuration of carbon atom 2. That the objections of Marvel and Noyes are not valid is readily seen from the following graphic expressions:



The configuration of carbon atoms 3, 4, and 5 are identical in the two diazo derivatives and if the configuration of carbon atom 2 in the acid derived from (I) is different from that derived from (III) then the resulting configurations of carbon atom 2 were brought about by other factors than the configuration of carbon atoms 3, 4, and 5. Hence any conclusions reached on the basis of experiments on one group of substances may be applied also to the other group.

In 1920-21 a brief report<sup>3</sup> was published on the diazo ester obtained from *d*-aspartic ester. The evidence contained in it is suggestive of the possibility of the existence of optically active diazosuccinic ester. The publication of the details was delayed, as it was our intention to postpone it until the preparation of a crystalline aliphatic diazo derivative was accomplished. External consideration, however, prompts us to publish the details of the work on the diazodiethyl succinates at this date.

The results of the analysis in the previous communication and the optical rotation of the diazo esters have already been reported. The substance was prepared by the method of Curtius and purified by distillation at a pressure of not more than 0.1 mm. By further fractionation it was not possible to separate the material into

<sup>3</sup> Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1920-21, xlv, 593.

fractions differing in their optical activity. The highest optical activity observed was  $[\alpha]_D^{20} = 1.34^\circ$ .

It was not excluded that the substance was a mixture of the *d* and *dl* forms. There was, in fact, evidence for the assumption that the material is easily inactivated. Thus in some experiments the diazo esters could not be distilled unless previously dried by barium oxide. On such treatment the product generally darkened and on distillation yielded a diazo ester of much lower optical activity than the samples which did not require the treatment with barium oxide.

In order to find more rigorous proof for the existence of an optically active diazodiethyl succinate, the material was converted into diethyl malate. Since the optical rotation of the unracemized diazo derivative is not known and since the specific rotation of diethyl malate is much higher than the one observed for the diazo derivative, it was reasoned that an increase in optical activity after hydrolysis should be interpreted in the sense of the conversion of diazo derivative into an optically active hydroxy ester. The result of the experiment seemed to point in the desired direction, since the rotation of the material obtained from hydrolysis of the diazo ester was higher than the rotation of the original material. However, the increase was not sufficiently great to render the evidence conclusive.

In view of this, the diazo ester was converted into the halogen acid esters by the action of hydrobromic or hydrochloric acid. If under such conditions active halogen succinic esters were obtained this finding could be interpreted only in the sense of the existence of an optically active diazo ester. Optically active esters of the chloro- and bromosuccinic esters were prepared. The analysis of the bromo derivative was quite satisfactory; the theory requiring Br = 31.60 per cent and the value found being Br = 31.12 per cent. The optical rotation of the material was  $[\alpha]_D^{20} = + 0.40^\circ (\pm 0.00)$ .

The chloroethyl succinate analyzed as follows:

Calculated.	C	46.11,	H	6.24,	Cl	17.01.
Found.	"	46.24,	"	7.04,	"	15.35.

The optical rotation was  $[\alpha]_D^{20} = + 0.86^\circ (\pm 0.00)$ . The low value of chlorine can be explained by the presence in the material

of either malic or fumaric esters. The material was therefore hydrogenated in the presence of colloidal palladium and it absorbed an amount of hydrogen which would be required by the presence of 10.23 per cent of fumaric acid. Thus the discrepancy between the theoretical and found values of chlorine may be explained on the basis of the presence of fumaric acid, in which case the chloromalic acid is optically active.

Thus, all evidence points towards the existence of optically active diazodiethyl succinate. However, further evidence is required for the final solution of the problem. Work in this direction is in progress.

#### EXPERIMENTAL.

*Nitrogen Estimation.*—It was found convenient to use Van Slyke's amino apparatus. For this purpose a leveling bulb was connected to the lower outlet of the mixing chamber by a rubber tube, and filled with mercury. Before beginning the analysis the mixing chamber is filled with mercury. By lowering the leveling bulb a vacuum is created. 25 per cent sulfuric acid is then introduced into the mixing chamber and then the diazo derivative dissolved in a mixture of equal parts of isopropyl alcohol and water. On gentle shaking the operation is completed in about 2 minutes. The further procedure is the same as in Van Slyke's method for amino nitrogen estimation.

By this method diazoethyl acetate and diazoethyl succinamide were analyzed with the following results:

2 cc. solution of 0.2990 gm. diazoethyl acetate in 10 cc. isopropyl alcohol and water 1:1 gave in Van Slyke apparatus 1.11 cc.  $N_2$ ,  $P = 770.8$  mm.,  $t = 23^\circ C$ .

$C_4H_6O_2N_2$ .	Calculated.	N	20.74.
	Found.	"	21.10.

2 cc. solution of 0.7200 gm. diazoethyl succinamide in 10 cc. water gave in Van Slyke apparatus 2.09 cc.  $N_2$ ,  $P = 762.2$  mm.,  $t = 24^\circ C$ .

$C_6H_8O_3N_2$ .	Calculated.	N	16.43.
	Found.	"	16.24.

To test further this method of analysis, on several occasions the analysis was controlled by an estimation by the Dumas method. For the latter purpose the substance was weighed in a very fine capillary and the further analysis was completed in the usual way. The results of the comparative analyses were as follows.

2 cc. solution of 0.6760 gm. diazodiethyl succinate in 10 cc. 1:1 isopropyl alcohol gave in Van Slyke apparatus 1.60 cc.  $N_2$ ,  $P = 764$  mm.,  $t = 24^\circ C$ .  
 0.0978 gm. substance gave, Dumas, 12 cc.  $N_2$ ,  $P = 764$  mm.,  $t = 26^\circ C$ .

$C_8H_{12}O_4N_2$ . Calculated.	N	14.00.
Found (Van Slyke).	"	13.93.
" (Dumas).	"	14.07.

*Preparation of Diazodiethyl Succinate.*—Much difficulty was experienced in the purification of this ester, as the least trace of water or some other impurity seemed to interfere with distillation. Methods of treatment had to be changed at times to make distillation possible. Some of these will be described below.

60 gm. of diethyl aspartate hydrochloride ( $[\alpha]_D^{20} = +8.15^\circ$ ) were dissolved in 90 cc. of water and cooled to  $0^\circ$ . To this was added a solution of 45 gm. of sodium nitrite in 90 cc. of water and also about 200 cc. of ether. The mixture was thoroughly cooled in a freezing mixture (salt and ice), whereupon cold 25 per cent sulfuric acid was gradually added. The ether layer was drained off, more ether was added, which was followed by the addition of more acid. This procedure was repeated until the ether extract was practically colorless. The ether extract was then washed with a cold saturated solution of sodium carbonate until the aqueous layer, which in the first washing turned red, remained practically colorless. The ether solution was then washed with distilled water and dried by allowing it to stand over anhydrous sodium sulfate for several hours.

The ether was subsequently removed by distilling under reduced pressure. An attempt to distill the residue was unsuccessful since the high vacuum could not be maintained. The ester was therefore redissolved in ether and treated with anhydrous sodium sulfate. After about 2 hours the ether was removed and the residue distilled. Two fractions were obtained, boiling at  $75-80^\circ C$ ., at a pressure of about 0.12 mm.

2 cc. solution of 0.0984 gm. Fraction I in 10 cc. isopropyl alcohol and water 1:1 gave 2.35 cc.  $N_2$ ,  $P = 766.4$  mm.,  $t = 21^\circ C$ .

2 cc. solution of 0.0714 gm. Fraction II in 10 cc. isopropyl alcohol and water 1:1 gave 1.78 cc.  $N_2$ ,  $P = 766.4$  mm.,  $t = 21^\circ C$ .

	$N_2$ per cent	Impurity. per cent
$C_8H_{12}O_4N_2$ . Calculated.	14.00	
Found, Fraction I.	13.64	2.5
" " II.	14.20	0.0

$$\text{Fraction I. } [\alpha]_D^{20} = \frac{+0.33^\circ \times 100}{1 \times 32.9} = +1.00^\circ$$

$$\text{Fraction II. } [\alpha]_D^{20} = \frac{+0.47^\circ \times 100}{1 \times 35.1} = +1.34^\circ$$

In subsequent preparations of the pure diazo derivative it was found impossible to distill the material without further treatment. In some cases it was found necessary to treat it with anhydrous potassium carbonate. In other instances this did not suffice but a further treatment with calcium hydroxide was necessary. Barium oxide was also used with success. In the experiments in which calcium hydroxide was used the optical activity of the pure diazodiethyl succinate was found to be

$$[\alpha]_D^{20} = \frac{+0.22^\circ \times 100}{0.5 \times 100} = +0.44^\circ$$

Found. N 14.26 and 14.20 per cent.

In another experiment in which calcium hydroxide was used the optical activity was found to be

$$[\alpha]_D^{20} = \frac{+0.25^\circ \times 100}{0.5 \times 100} = +0.50^\circ$$

Found. N 14.04 per cent.

*Conversion of Diazodiethyl Succinate into Hydroxy Succinate.*—About 5 gm. of pure diazodiethyl aspartate were dissolved in 50 cc. of ether and cooled by means of a freezing mixture. 25 per cent sulfuric acid was then gradually added, with shaking, until the yellow color disappeared. The ether layer was separated, and the mother liquor extracted with ether several times. The ether extracts were combined, dried with sodium sulfate, and the ether was removed under diminished pressure. The residue weighed 3.01 gm. This was diluted to 10 cc. with ether and optical activity determined,

$$[\alpha]_D^{20} = \frac{+0.16^\circ \times 100}{1 \times 30.1} = +0.53^\circ$$

The reading of the original diazo derivative without solvent was

$$[\alpha]_D^{20} = \frac{+0.22^\circ \times 100}{0.5 \times 100} = +0.44^\circ$$

Diazo nitrogen in the original diazo derivative, as already mentioned, was  $N = 14.26$  and  $14.20$  per cent.

In another experiment a larger quantity of diazo ester was prepared and hydrolyzed, without previous distillation. The original material had an optical rotation  $[\alpha]_D^{20} = +3.84^\circ$ . The ethyl malate was prepared in the usual way and the product fractionated as follows:

5.82 gm. Fraction I, boiling at  $114-117^\circ\text{C.}$ , made up to 10 cc. with ether gave

$$[\alpha]_D^{20} = \frac{+1.11^\circ \times 100}{1 \times 58.2} = +1.90^\circ$$

5.36 gm. Fraction II, boiling at  $127-136^\circ\text{C.}$ , made up to 10 cc. with ether gave

$$[\alpha]_D^{20} = \frac{+1.60^\circ \times 100}{1 \times 53.6} = +2.98^\circ$$

4.07 gm. Fraction III, boiling at  $136-160^\circ\text{C.}$ , made up to 10 cc. with ether gave

$$[\alpha]_D^{20} = \frac{+2.88^\circ \times 100}{1 \times 40.7} = +7.07^\circ$$

This material contained in addition to the diethyl malate, partly hydrolyzed material and some fumaric ester, which had been isolated on many occasions. The partial saponification was made evident on another material which happened to have a lower optical rotation. The fractions obtained on first distillation showed values for carbon which were too low for the esters, which could be explained on the basis of partial saponification. After several fractionations a product was obtained which analyzed correctly for diethyl malate, as follows:

0.1006 gm. substance: 0.1854 gm.  $\text{CO}_2$  and 0.0670 gm.  $\text{H}_2\text{O}$ .

$\text{C}_8\text{H}_{10}\text{O}_6$ . Calculated. C 50.52, H 7.37.

Found. " 50.26, " 7.45.

*Conversion of Diazodiethyl Succinate into Monobromodiethyl Succinate.*—180 gm. of diethyl aspartate hydrochloride were diazotized as in the preparation of the diazo derivative. The diazo ester was washed with a solution of sodium carbonate and dried over sodium sulfate. Without further purification the ethereal solution of the diazo ester was cooled and then slowly added to

a cold saturated solution of hydrobromic acid gas in ether. After the evolution of gas had ceased the reaction mixture was of a dark brown color which again turned to straw yellow on washing with an aqueous sodium carbonate. The ethereal solution was then washed with distilled water, dried over sodium sulfate, and the ether removed under diminished pressure. The residue was then subjected to fractional distillation:

Fraction No.	Boiling point.	Pressure.	$[\alpha]_D^{20}$	Br content.
	$^{\circ}\text{C.}$	$\text{mm.}$		<i>per cent</i>
I	75-80	0.134	$\frac{+0.22^{\circ} \times 100}{0.5 \times 100} = +0.44^{\circ}$	Not determined.
II	76-79	0.067	$\frac{+0.25^{\circ} \times 100}{0.5 \times 100} = +0.50^{\circ}$	28.40
III	77-79	0.040	$\frac{+0.24^{\circ} \times 100}{0.5 \times 100} = +0.48^{\circ}$	30.10
IV	75-77	0.067	$\frac{+0.20^{\circ} \times 100}{0.5 \times 100} = +0.40^{\circ}$	30.56
V	74-76	0.094	$\frac{+0.16^{\circ} \times 100}{0.5 \times 100} = +0.32^{\circ}$	30.51

Fractions IV and V were combined and redistilled:

Fraction No.	Boiling point.	Pressure.	$[\alpha]_D^{20}$	Br content.
	$^{\circ}\text{C.}$	$\text{mm.}$		<i>per cent</i>
I	66-70	0.037	$\frac{+0.20^{\circ} \times 100}{0.5 \times 100} = +0.40^{\circ}$	30.36
II	68-70	0.023	$\frac{+0.20^{\circ} \times 100}{0.5 \times 100} = +0.40^{\circ}$	30.99
III	69-70	0.023	$\frac{+0.20^{\circ} \times 100}{0.5 \times 100} = +0.40^{\circ}$	30.74
IV	69-70	0.023	$\frac{+0.20^{\circ} \times 100}{0.5 \times 100} = +0.40^{\circ}$	30.51

Fractions II and III were combined and redistilled:

Fraction No.	Boiling point.	Pressure.	$[\alpha]_D^{20}$	Br content.
	$^{\circ}\text{C.}$	$\text{mm.}$		<i>per cent</i>
I	80-81	0.040	$\frac{+0.20^{\circ} \times 100}{0.5 \times 100} = +0.40^{\circ}$	30.82
II	81-82	0.046	$\frac{+0.20^{\circ} \times 100}{0.5 \times 100} = +0.40^{\circ}$	31.12
III	81-82	0.047	$\frac{+0.20^{\circ} \times 100}{0.5 \times 100} = +0.40^{\circ}$	30.68

0.2058 gm. substance: 0.1500 gm. AgBr.

$\text{C}_8\text{H}_{13}\text{O}_4\text{Br}$ . Calculated. Br 31.60.

Found. " 31.12.

*Conversion of Diazodiethyl Succinate into Monochlorodiethyl Succinate.*—Diazoethyl succinate was dissolved in 5 volumes of dry ether and the solution treated with dry HCl gas with cooling. After the evolution of nitrogen had ceased the ether was distilled off under reduced pressure and the optical activity of the residue determined. It was found to be:

$$[\alpha]_D^{20} = \frac{+0.36^{\circ} \times 100}{0.5 \times 100} = +0.72^{\circ}$$

The original diazo derivative showed:

$$[\alpha]_D^{20} = \frac{+0.25^{\circ} \times 100}{0.5 \times 100} = +0.50^{\circ}$$

The chlovester was fractionated as follows:

Fraction No.	Boiling point.	Pressure.	$[\alpha]_D^{20}$
	$^{\circ}\text{C.}$	$\text{mm.}$	
I	61-68	0.050	$\frac{+0.18^{\circ} \times 100}{0.5 \times 100} = +0.36^{\circ}$
II	67-68	0.048	$\frac{+0.25^{\circ} \times 100}{0.5 \times 100} = +0.50^{\circ}$
III	68-70	0.057	$\frac{+0.43^{\circ} \times 100}{0.5 \times 100} = +0.86^{\circ}$



Fraction III was analyzed:

0.1004 gm. substance: 0.0580 gm.  $\text{H}_2\text{O}$  and 0.1900 gm.  $\text{CO}_2$ .

$\text{C}_8\text{H}_{13}\text{O}_4\text{Cl}$ . Calculated. C 46.11, H 6.24, Cl 17.01.

Found. " 46.18, " 6.47, " 15.35.

0.5602 gm. Fraction III was dissolved in ether and reduced with hydrogen in the presence of colloidal palladium. The reduction was continued until no more hydrogen was being absorbed. 8.00 cc. hydrogen were absorbed.  $t = 22^\circ\text{C}$ .,  $P = 752$  mm.

The amount of hydrogen absorbed corresponded to 10.23 per cent of fumaric ester.

## STUDIES OF ACIDOSIS.

### XVIII. DETERMINATION OF THE BICARBONATE CONCENTRATION OF THE BLOOD AND PLASMA.

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(Received for publication, April 15, 1922.)

In order to determine the acid-base balance of the blood as outlined previously (Van Slyke, 1921) it is desirable to ascertain the bicarbonate concentration existing in the blood in the body. As pointed out in the above mentioned paper,<sup>1</sup> determinations of the CO<sub>2</sub> content of the whole blood (Peters and Barr, 1920-21) or plasma (Van Slyke and Cullen, 1917), after equilibration at an arbitrarily chosen CO<sub>2</sub> tension, or determinations of the plasma bicarbonate by titration to a constant pH as end-point (Van Slyke, Stillman, and Cullen, 1919), although adequate to indicate alkali deficit or excess, are not sufficient to indicate conditions in which the respiratory control of the CO<sub>2</sub> tension also is disturbed, and in consequence the blood pH is varied from the normal.

To determine the bicarbonate of the blood under the conditions existing *in vivo* we may proceed in either of two ways. We may determine the total CO<sub>2</sub> (BHCO<sub>3</sub> + H<sub>2</sub>CO<sub>3</sub>) of either blood or plasma, together with the pH, and estimate the BHCO<sub>3</sub> by the equation,  $\text{BHCO}_3 = K' \frac{\text{H}_2\text{CO}_3}{[\text{H}^+]}$ ; or we may titrate the plasma as described by Van Slyke, Stillman, and Cullen (1919), with the modification that the end-point of the titration is the pH of the individual plasma as drawn, rather than the constant pH 7.4 used by the above authors.

#### *Gasometric Determination of Plasma of Whole Blood Bicarbonate.*

The total CO<sub>2</sub> is determined in either plasma or whole blood (Van Slyke and Stadie, 1921). The plasma pH is deter-

<sup>1</sup> Van Slyke (1921), pp. 171 and 172.

mined either electrometrically or by the colorimetric method of Cullen (1922). The bicarbonate is then calculated by the factor given in Table I. The factors in Table I are calculated from the mass action equation,  $[H^+] = 'K \frac{H_2CO_3}{BHCO_3}$ , transformed as follows. Expressing the total  $CO_2$  concentration as  $[CO_2]$ , the free dissolved  $CO_2$  as  $[H_2CO_3]$ , the bicarbonate as  $[BHCO_3]$ , we have Equation 1, which is L. J. Henderson's expression of the mass law as applied to  $BHCO_3 + H_2CO_3$  solutions (1909). Equations 2

TABLE I.

pH	Total $CO_2$ in the form of bicarbonate.	
	Whole blood.	Plasma or serum.
	<i>per cent</i>	<i>per cent</i>
7.0	87.6	88.5
7.1	89.9	90.9
7.2	91.8	92.7
7.3	93.4	94.1
7.4	94.8	95.3
7.5	95.8	96.2
7.6	96.6	97.0
7.7	97.3	97.6
7.8	97.8	98.1

and 3 are the intermediate steps leading to Equation 4.  $pK'$  is the negative logarithm of  $K'$ , as pH is of  $H^+$ .

$$(1) \quad [BHCO_3] = K' \frac{[H_2CO_3]}{[H^+]}$$

$$(2) \quad [BHCO_3] = K' \frac{[CO_2] - [BHCO_3]}{[H^+]}$$

$$(3) \quad [BHCO_3] = \frac{1}{1 + \frac{[H^+]}{K'}} [CO_2]$$

$$(4) \quad [BHCO_3] = \frac{1}{1 + 10^{pK' - pH}} [CO_2]$$

$\frac{1}{1 + 10^{pK' - pH}}$  in Equation 4 is the fraction of the total  $CO_2$  in the form of bicarbonate.

The value of  $pK'$  was originally determined by Hasselbalch (1917) to be 6.03 for 0.05 M  $\text{NaHCO}_3$ , 6.06 for 0.03 M, and 6.08 for 0.02 M.<sup>2</sup> In whole blood Hasselbalch found that the same values held. Haggard and Henderson (1919) from a compilation of the data in the literature estimated the average value of  $K'$  for whole blood to be  $8 \times 10^{-8}$  whence  $pK' = 6.10$ . In work shortly to be reported from this laboratory the value of  $pK'$  has been found to be 6.15 for normal whole blood, 6.10 for plasma. From these values of  $pK'$  the values of  $\frac{100}{1 + 10^{pK' - pH}}$ , the percentage of total  $\text{CO}_2$  in the form of bicarbonate, given in Table I are calculated.

#### *Titration of Plasma Bicarbonate.*

This determination may be conveniently combined with the colorimetric method of Cullen (1922), since the diluted 1 cc. sample of plasma used in Cullen's pH determination serves to indicate the end-point in the bicarbonate titration. The oxalated blood is drawn and centrifuged under rigorous precautions to avoid loss of  $\text{CO}_2$  which are detailed by Cullen (1922) in the accompanying description of his colorimetric pH determination. 1 cc. of the plasma, to serve as a standard for the end-point, is treated exactly as described by Cullen to prepare it for colorimetric pH determination, by diluting it under oil, in a tube of 20 mm. diameter, with 20 cc. of neutral 0.9 per cent NaCl solution, containing 7 drops of 0.03 per cent phenol red.

Another 1 cc. sample of the plasma is transferred to a round flask of about 100 cc. capacity, 5 cc. of 0.01 N HCl, which is made up in neutral 0.9 per cent NaCl, are added, and the  $\text{CO}_2$  is removed by whirling the mixture about the flask for at least 1 minute, as described by Van Slyke, Stillman, and Cullen (1919). The solution is poured into a test-tube of the same diameter as that containing the standard, the portion adherent to the walls of the flask being transferred by rinsing with 10 cc. of 0.9 per cent NaCl divided into three portions. 7 drops of the 0.03 per cent phenol red

<sup>2</sup>The figures actually given by Hasselbalch for  $pK'$  are 0.3 higher, because he used the equivalent concentration of  $\text{H}_2\text{CO}_3$ , which is twice the molecular, in calculating the  $\text{BHCO}_3 : \text{H}_2\text{CO}_3$  ratio. When the molecular ratio employed by most other authors is used, Hasselbalch's data give the figures quoted.

solution are added, and 0.01 N NaOH is run in from a burette, which permits readings to 0.01 cc., until the color matches that of the standard. As the end-point is approached, sufficient 0.9 per cent NaCl is added to bring the volume to 20 cc.

The 0.01 N NaOH like the 0.01 N NaCl, is made up by diluting 1 volume of 0.1 N pure solution to 10 volumes with neutral CO<sub>2</sub>-free 1 per cent NaCl.

The use of saline solution instead of water has the advantage of preventing the formation of a permanent cloudy precipitate of globulin.

TABLE II.

Plasma No.	Titration method.		Gasometric method.				
	0.01 N HCl used in titrations.	BHCO <sub>3</sub> concentration.	Total CO <sub>2</sub> .	pH by Cullen's method.	Proportion of total CO <sub>2</sub> as BHCO <sub>3</sub> (from Table I).	BHCO <sub>3</sub>	
	cc.	milli-molecular	vol. per cent		per cent	vol. per cent CO <sub>2</sub>	milli-molecular
1	2.14						
	2.15	21.5	51.6	7.44	95.7	49.3	22.0
2	1.61						
	1.61	16.1	37.6	7.44	95.7	36.0	16.1
3	1.30						
	1.26	12.8	30.9	7.38	95.1	29.4	13.1
4	1.22						
	1.24	12.3	30.3	7.22	92.8	28.1	12.5

The precautions outlined by Van Slyke, Stillman, and Cullen (1919) and by Stillman (1919), in particular those concerning the avoidance of CO<sub>2</sub> in the standard NaOH solution, are to be observed.

The nature of the results obtained is indicated by Table II.

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## STUDIES OF ACIDOSIS.

### XIX. THE COLORIMETRIC DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF BLOOD PLASMA.\*

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(Received for publication, April 15, 1922.)

The electrometric method for determining the blood reaction requires relatively large quantities of blood, elaborate apparatus, constant attention, and extremely careful manipulation when dealing with CO<sub>2</sub>-containing solutions, and is, therefore, of only limited availability. The colorimetric methods so far reported have not been comparable in accuracy to the electrometric methods.

Levy, Rowntree, and Marriott (1915), added phenol red to the dialysate of the blood and matched the color produced against the color standards, thus avoiding the difficulties caused by the color of the blood as well as by the protein effect of the plasma proteins upon the dye. Although, because of several inherent errors, the method is not suited for exact determination of the actual reaction of the blood, the method has been of great value in affording a measure of the change in reaction.

The more important errors in this method were: (1) loss of carbon dioxide; and (2) the difference in the reaction of two solutions, one containing protein, when separated by a semipermeable membrane. This phenomenon, the Donnan "membrane equilibrium," has been recently studied by Loeb (1921-22). Minor errors involved were the variable dilutions and temperature errors.

Attempts to prevent the loss of CO<sub>2</sub> have been reported by Scott (1917) and more recently by Dale and Evans (1920-21). Dale and Evans enclosed their dialysis membrane in a CO<sub>2</sub>-tight con-

\* A preliminary report of this work appeared in the Proceedings of the American Society of Biological Chemists (*J. Biol. Chem.*, 1922, 1, p. xvii).



tainer. They used neutral red as an indicator, which was added to the dialysate after the dialyzing membrane had been removed and before the liquid paraffin had been added.

The technique for colorimetric pH determination has been greatly refined in recent years, especially by Walpole's (1914) introduction of the comparator and by Clark's (1920) studies, and it was felt that it should now be possible to develop an accurate colorimetric method for the determination of the pH of blood plasma. (Parsons (1919-20) has recently shown that when whole blood is used for the electrometric determination, the pH obtained is that of the plasma, the suspended corpuscles not affecting the result.) In an accompanying paper Cullen and Hastings (1922) show (contrary to the finding of Evans), that when proper precautions are observed, the colorimetric agrees exactly with the electrometric method in CO<sub>2</sub>-containing solutions as well as in phosphate solutions.

The method which has been developed and which will be described below eliminates the Donnan effect by determining the reaction of the diluted plasma itself, and allows for the protein, salt, dilution, and temperature effects, by a correction experimentally determined by comparison of colorimetric results with those by the standard electrometric method.

#### EXPERIMENTAL.

*Dilution Error.*—As is evident from Fig. 1, serum showed a continued increase in alkalinity when diluted with 0.9 per cent NaCl solution up to a dilution of 15- to 20-fold. Beyond 20-fold the change upon further dilution is so small as to be practically negligible. A dilution of 20-fold with 0.9 per cent saline solution was therefore chosen as optimal. A dilution as great as this has the further advantage of making it possible to work with as little as 0.5 to 0.25 cc. of serum, obtainable from 1 cc. of blood.

The use of salt solution instead of water as a plasma diluent is necessary because the curve is not only steeper with water than with saline solution, but also because with water globulin precipitation occurs before the plateau of the curve is reached.

Addition of neutral potassium oxalate to the blood up to 1 per cent does not effect the dilution curve.

*Temperature Effect.*—The effect of change in room temperature upon the colorimetric reading is shown in Fig. 2. This curve

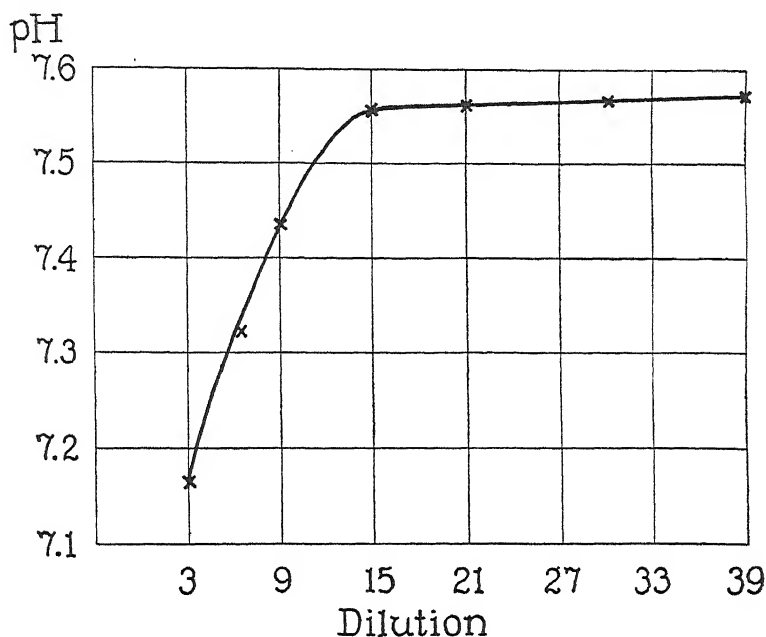


FIG. 1.

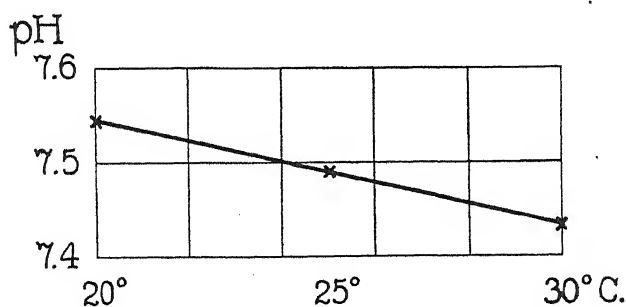


FIG. 2.

is identical with several obtained in dilute plasma solutions. Both the unknown and the phosphate standards containing phenol red were at the temperature indicated.

The temperature correction is + 0.01 pH per increase of 1°C.

*Hemolysis.*—Slight hemolysis does not appear to interfere appreciably with the reading, but with any marked hemolysis

TABLE I.

*Comparison of Colorimetric (20°) and Electrometric (38°) pH Values of Horse Plasma.*

No.	Total CO <sub>2</sub> content.	CO <sub>2</sub> tension.	Colorimetric 20°C.	Electrometric 38°C.	Colorimetric at 20° minus electrometric at 38°	Deviation from average difference.
	<i>millimols</i>	<i>mm. Hg</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>
1	28.0		7.57	7.42	0.15	+0.03
2	27.7	49.5	7.52	7.39	0.13	+0.01
3	28.5		7.53	7.41	0.12	0.00
4	27.2	44.7	7.53	7.38	0.15	+0.03
5	26.7		7.55	7.43	0.12	0.00
6	27.1	44.5	7.54	7.42	0.12	0.00
7	27.9		7.50	7.42	0.08	-0.04
8	28.0	20.0	7.73	7.63	0.10	-0.02
9	25.9	38.7	7.57	7.45	0.12	0.00
10	29.3	57.9	7.45	7.33	0.12	0.00
11	18.1	19.2	7.72	7.57	0.15	+0.03
12	24.5	45.6	7.48	7.35	0.13	+0.01
13	20.3	19.4	7.69	7.63	0.06	-0.06
14	26.7	45.6	7.51	7.40	0.11	-0.01
15	27.7	47.3	7.51	7.40	0.11	-0.01
Average for plasma.....					0.12	±0.02

The values in Tables I and II represent the electrometric reading at 38°C. on undiluted plasmas and sera of bloods equilibrated at 38° at the indicated CO<sub>2</sub> tensions as compared with the colorimetric readings corrected to 20°C. of 20-fold dilution with saline solution of the same plasma or serum. Where no CO<sub>2</sub> tension is given the determination was carried out on the plasma of the blood as drawn. The total CO<sub>2</sub> content is given to show there is no relationship between the CO<sub>2</sub> content and the deviation figure.

the change in the tone of the dye is so great that accurate readings are impossible. Since serum may be used instead of plasma, oxalate may be dispensed with when it causes hemolysis.

*Total Correction.*—Since the primary interest in the reaction of the blood is in the reaction at body temperature it was decided to refer all colorimetric readings made at room temperature to the electrometric value at 38°. Simultaneous determinations, electrometric at 38° on undiluted plasma and colorimetric at room temperature on 20-fold dilute plasma, were carried out on a series of bloods which had previously been equilibrated with gas mixtures of varying CO<sub>2</sub> tension. The results of twenty-three such determinations on horse blood are given in Tables I and II. The colorimetric determinations are all corrected to 20°C., i.e.,  $pH_{20^\circ} = pH_t + 0.01(t^\circ - 20^\circ)$  where  $t^\circ$  = room temperature.

TABLE II.

*Comparison of Colorimetric (20°) and Electrometric (38°) pH Values of Horse Serum.*

No.	Total CO <sub>2</sub> content.	CO <sub>2</sub> tension.	Colorimetric 20°.	Electrometric 38°.	Colorimetric at 20° minus electrometric at 38°	Deviation from average difference.
	millivolts	mm. Hg	pH	pH	pH	pH
1	29.96		7.60	7.46	0.14	-0.02
2	22.1	17.9	7.81	7.65	0.16	0.00
3	27.51	38.8	7.65	7.47	0.18	+0.02
4	31.28	57.9	7.50	7.36	0.14	-0.02
5	29.9		7.58	7.45	0.13	-0.03
6	28.4	45.6	7.58	7.41	0.17	+0.01
7	28.7	45.6	7.59	7.43	0.16	0.00
8	28.8	45.6	7.59	7.43	0.16	0.00
Average for serum.....					0.16	±0.02

It is apparent that there is a distinct difference between the correction for plasma and that for serum. The same difference was found in another series of twenty-two sera and fourteen plasmas. These determinations were made on the same horse and it is felt that the difference between the plasma and serum correction must be due to the presence of the fibrin in the plasma, since the addition of neutral potassium oxalate to defibrinated blood or to the separated plasma or serum in concentration up to 1 per cent does not affect either the electrometric reading or the colorimetric reading on the diluted serum or plasma. Moreover, the same difference is noted when the plasma and serum are ob-

tained from different portions of the same sample of blood where they have the same actual (electrometric) pH. The constancy, however, of the correction value for the fifteen plasmas at 0.12 pH and of the eight sera at 0.16 pH is striking.

In addition to the difference between the plasma and serum of horse blood, it has been found that the same fluid (*e.g.*, plasma) has different correction values in different species. In Table III the extent of this variation is indicated. It is possible also that in some pathological conditions the difference between the electrometric and colorimetric pH reading may be altered. This

TABLE III.

*Average Values of Correction for Different Systems.*

Number of specimens in series.	System.	Average correction.	Average deviation.	Maximum deviation.
10	Human plasma.	0.23	0.02	0.04
5*	Human plasma.	0.22	0.00	0.01
7*	Rabbit plasma.	0.17	0.01	0.02
4	Dog serum.	0.35		0.03
9†	Human joint fluid.	0.21	0.026	0.05

\*These five human plasma values and seven rabbit plasma values were furnished by Dr. A. B. Hastings (personal communication).

†Boots and Cullen (1922).

question is being studied further. The data given for the correction for colorimetric pH readings in fluids other than human plasma are too few in number to establish reliable values for the correction in these instances, but serve to indicate its nature.

However, the seventeen values for human plasma, which were obtained from seventeen individuals, indicate that the value 0.22 is accurate to within the combined error 0.04 pH of the two methods.

*Calculation.*—The calculation of the reaction of the blood at 38° is as follows:  $\text{pH}_{38^\circ} = \text{pH}_{\text{colorimetric } 20^\circ} - C$ , where C is the appropriate correction for the system under investigation. If it is

not feasible to make the determination at 20°C. the colorimetric reading for horse or human blood may be corrected as indicated above.

$$\text{Colorimetric pH}_{20^{\circ}} = \text{colorimetric pH}_{t^{\circ}} + 0.01 (t^{\circ} - 20^{\circ}).$$

Although this temperature correction appears accurate for the range 20–30°, because of the empirical nature of the correction, it is desirable to make all determinations at 20°. This is especially true in using the method with other than horse or human blood. For values reported above, the room temperatures were used either at 20° or between 20 and 24°.

*Application of the Method.*—The reliability of the colorimetric method in its present state of development should be considered from two distinct view-points.

1. *Determination of Relative pH Changes.*—In studying the changes in reaction which may occur during the course of an experiment or in following the condition of a patient, for example, under alkali therapy, the proposed method can be used with an accuracy of at least  $\pm 0.04$  pH and probably with an accuracy of  $\pm 0.02$ . Whether or not extreme pathological conditions change the correction remains to be determined.

2. *Determination of Absolute pH Values.*—The variations in the correction factors which are given in Table III (from 0.11 pH for horse plasma to 0.35 pH for dog serum) make it necessary to determine the correction factor for each particular species. However, the individual variations from the average corrections in the cases of human and horse plasma and horse serum are so small as to make it appear probable that the species corrections determined are approximately constant, and that the method can be used with an accuracy of about  $\pm 0.04$ . The method has already proved of clinical value in detecting gross deviations (0.2 to 0.3 pH) from the normal in conditions such as nephritic acidosis.

#### *Details of Determination.*

*Blood Sampling.*—The blood is drawn without stasis and without exposure to air into a glass syringe or tube coated with potassium oxalate to make 0.3 per cent and containing mineral oil. (Mineral oil, potassium oxalate, and glassware must be

tested for neutrality.) Then, without exposure to the air, the blood is run into a tube under oil to the complete filling of the tube. A one-hole rubber stopper is slipped into the tube, expelling through the hole the oil that remains over the blood. The hole is closed with a glass plug, the tube is placed in a centrifuge, and whirled. The plug is then taken out and as the stopper is removed from the tube, oil is allowed to run in through the hole in the stopper to cover the surface of the plasma so that it is never exposed to air. The plasma is then transferred under oil to another tube. Simply covering the blood with oil is not sufficient to prevent the loss of  $\text{CO}_2$  during centrifuging. Oil suffices to prevent loss of  $\text{CO}_2$  from solutions which are standing quietly for short periods only.

*Determination.*—35 drops of 0.03 per cent phenol red solution are added to 100 cc. of 0.9 per cent sodium chloride solution, freshly prepared from redistilled water. 1 to 3 drops of 0.02 N NaOH are added to bring this solution to pH 7.4 or 7.5. 20 cc. portions are then placed in suitable tubes (see below) and covered with mineral oil. Other tubes are prepared with 20 cc. saline solution without indicator.

A 1 cc. portion of the plasma is then allowed to run under the oil into the indicator-saline solution and another 1 cc. portion into the 20 cc. of saline solution. This latter tube is for use with the pH standard in the comparator. A 1 cc. bulb pipette, graduated to deliver between two marks, is convenient as it is otherwise difficult to deliver all the plasma from the tip of a pipette which is under a layer of oil. The plasma and saline solution are then mixed by introducing a stirring rod through the oil, and the pH determination is made by placing the tubes in a comparator block, and matching to the nearest standard color tube. It is possible to read to 0.01 or 0.02 pH. The temperature of the solution is determined by inserting a thermometer into the solution immediately after the pH reading. The reading should, if possible, be made at 20°. This can be conveniently accomplished by placing the diluted plasma tubes together with the necessary standard tubes in a large beaker of water at 20°. When they have attained 20° they are removed, and placed in the comparator. The reading is easily made before significant temperature change occurs. The pH observed is corrected to 38° by the formula given above.

*Indicator Solution.*—5 drops of 0.03 per cent phenol red solution are added to 15 cc. of the standard solution. The concentration of dye required varies somewhat with different lots. It is best to prepare a concentrated stock solution, and determine by experiment the dilution required to give satisfactory depth of color over the desired pH range. The indicator solution must be neutral. After the addition of 1 drop to 3 cc. of redistilled water, the water must not be red.

*Standards.*—Sørensen's phosphate standards are prepared from Merck's special reagents in steps of pH 0.05 from about pH 7.2 to 7.7. The M/15 phosphate solution should be prepared from special reagent salts (Merck's are satisfactory) by dissolving the following quantities to a liter with distilled water:

	<i>gm.</i>
$\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ "Sørensen salt" .....	11.87
$\text{Na}_2\text{HPO}_4$ anhydrous (Merck) .....	9.47
$\text{KH}_2\text{PO}_4$ .....	9.08

The proportions of acid and alkaline phosphates are given in Table IV. These mixtures may be kept for some weeks in Pyrex glass in the refrigerator.

TABLE IV.  
*Phosphate Mixtures (Phenol Red Range).*

pH	M/15 $\text{Na}_2\text{HPO}_4$	M/15 $\text{KH}_2\text{PO}_4$
	<i>cc.</i>	<i>cc.</i>
7.0	61.1	38.9
7.05	63.9	36.1
7.10	66.6	33.4
7.15	69.2	30.8
7.20	72.0	28.0
7.25	74.4	25.6
7.30	76.8	23.2
7.35	78.9	21.1
7.40	80.8	19.2
7.45	82.5	17.5
7.50	84.1	15.9
7.55	85.7	14.3
7.60	87.0	13.0
7.65	88.2	11.8
7.70	89.4	10.6
7.75	90.5	9.5
7.80	91.5	8.5



The color standards contain phenol red and must be renewed or checked against a fresh tube of dye at least once every week; as there is a slow fading of color.

*Apparatus.*—The comparator required is conveniently made from a block 3 × 6 inches. The holes for the tubes are of 1 inch diameter. The slits for the light are best made by boring two  $\frac{1}{2}$  inch holes and gouging out the remaining wood with a chisel. The tubes must be of clear, non-alkaline glass of *uniform* diameter. Tubes 20 mm. in diameter are convenient.

*Light.*—Either daylight or "Daylite" lamps are satisfactory.

*Test for Neutrality.*—The redistilled water is usually about pH 6.2 to 6.5. The easiest test is that of using both phenol red and methyl red. The water should give no (red) color with either indicator.

The syringe, pipettes, and tubes should be rinsed with redistilled water and dried. Syringes, tubes, and pipettes, washed and sterilized in the usual manner prevalent in bacteriological laboratories, are often dried from alkaline water.

The saline solution must be adjusted to pH 7.4 as described above.

The oxalate, when dissolved in water to a 0.5 per cent solution, should not be more alkaline than pH 7.2 to 7.4. The oil is tested by shaking with water containing phenol red and methyl red. The water must remain neutral.

### Methods.

*CO<sub>2</sub> Equilibration.*—About 50 cc. of the blood to be equilibrated were placed in an 800 cc. tonometer. The tonometer was partially evacuated and the calculated amount of CO<sub>2</sub> was then added from a burette. The tonometer was then equilibrated in a bath at 38°. The formula used for the calculation where the tonometer is not equilibrated to atmosphere after the attainment of the temperature (*i.e.* constant volume) is

$$\text{cc. CO}_2 = \frac{p_{38^\circ}\text{CO}_2 \times T_{\text{burette}} \times (V - s)}{311 \times (B - w)}$$

where  $p_{\text{CO}_2}$  = tension CO<sub>2</sub> in mm.

$T$  = absolute temperature.

$V$  = volume of tonometer.

$s$  = volume of solution.

$B$  = barometric pressure.

$w$  = vapor pressure.

The details of technique and the gas manifold used are described in detail elsewhere (Austin, Cullen, Hastings, McLean, Peters, and Van Slyke<sup>1</sup>). After equilibration, without loss of CO<sub>2</sub>, the blood was transferred to a second tonometer and again equilibrated to the same CO<sub>2</sub> tension. The solution was then transferred to a sampling tube over mercury from which samples were removed for colorimetric and electrometric determinations.

*Colorimetric Determination.*—The colorimetric readings were made in clear glass tubes of uniform bore (20 mm.). Redistilled water was used in making the 0.9 per cent saline solution and only fresh solutions were used. (The amount of CO<sub>2</sub> taken up by the saline solution upon standing may be sufficient to affect the reading.) An absolute guarantee of neutrality is conveniently attained by adding the 35 drops of dye to 100 cc. of saline solution and adjusting with 1 or 2 drops of 0.02 M NaOH to a pH of about 7.4 before measuring the 20 cc. portion into the tubes.

The readings were carried out in a comparator block using either daylight or the "Daylite" glass of the Nela Electric Co. Sunlight must be avoided. If turbid the plasma tubes should be placed alongside each other and next to the light.

All the measurements reported above were made in duplicate tubes. On each duplicate two or three readings were taken by each of two observers. It has been found desirable to make two or three rapid readings rather than strain the eyes by one prolonged comparison. The two observers always agreed within 0.01 pH.

The temperature of the colorimetric reading was obtained by placing a thermometer in the solution immediately after the reading.

The proportions of acid and alkaline phosphate given above were obtained from a large curve constructed by the use of a flexible spline from Sørensen's corrected figures. The color standards fade during the course of a week from 0.02 to 0.04 pH and it has been found convenient to make a quick comparison against the old standards and then to add dye to the fresh portion of standards with pH value on either side of the trial reading. This takes only a moment since the phosphate without dye keeps for a considerable period.

<sup>1</sup> To be published in a later number of this *Journal*.

*Electrometric Measurement.*—The determinations were carried out in a constant temperature room at 20° or in a thermostat maintained at 38°. Clark electrode vessels of 2 cc. capacity and saturated calomel electrodes with a saturated KCl bridge were used. For plasma determination a mixture of hydrogen and CO<sub>2</sub> at the same CO<sub>2</sub> tension at 38° as that used in equilibration was run into the electrode chamber which had previously been washed with water and filled with hydrogen. The CO<sub>2</sub> tension was calculated on the basis of expansion under atmospheric pressure since the readings were made with the cocks open so that the hydrogen was at atmospheric pressure. The formula used was

$$\text{cc. CO}_2 = \frac{p\text{CO}_2 \times V}{(B - w)}$$

$p\text{CO}_2$  = tension CO<sub>2</sub> desired at 38°

$w$  = vapor pressure at 38°

$V$  = volume of tonometer.

$B$  = barometric pressure.

After the initial reading, the plasma in the cell was replaced with another portion of plasma under the same bubble of hydrogen + CO<sub>2</sub> in the manner used by Hasselbalch to bring the hydrogen atmosphere to the same CO<sub>2</sub> tension as the solution. If the second reading checked with the first to within 1 millivolt, it was used as the correct reading. If it did not agree with the first refilling, the refilling and determination were repeated.

Our results agree with those of Hasselbalch in that, even when the electrode contained pure hydrogen at the start, only one or two refillings of blood are necessary to attain equilibration. The buffer value of plasma, however, is so low that if pure hydrogen is used five or six more refillings are necessary. It is possible, however, to get satisfactory results when the CO<sub>2</sub> tension is not known if the CO<sub>2</sub> tension in the hydrogen is approximately that of the plasma. For normal blood this may be taken as 40 mm. of Hg. Two or three refillings will then give constant readings. The measurements reported above, used in establishing the correction factors for the colorimetric method, are all made upon solutions equilibrated at known tensions.

*Standardization.*—For reasons which will be discussed more fully in a subsequent note by Cullen and Hastings, the electro-

metric determinations were standardized against 0.1 N HCl prepared by the method of Hulett and Bonner (1909) from constant boiling acid. We have assigned to it the values, derived from Noyes and Ellis (1917) for the activity coefficient, namely pH = 1.085 at 20° and pH = 1.090 at 38°, and have assumed that the temperature change of the activity coefficient is a linear function of the temperature. We have used the saturated calomel electrode without correction for diffusion potential (See Fales and Mudge, 1920).

Our procedure was to determine the "e"  $\left( \text{pH} = \frac{\text{E.M.F.} - "e"}{0.0001984 T} \right)$  of the entire system, including calomel cell and platinum electrode against 0.1 N HCl. One system was maintained at 38°, another at 20°. Using the values for "e" thus obtained—phosphate mixtures "7.4" were standardized against the 0.1 N HCl. This standard phosphate solution was then used before and after each set of plasma determinations to reestablish the "e" of the system. If the "e" changed during a determination that determination was rejected. By this technique the danger of the slow liberation of acid absorbed by the platinum black is minimized. The phosphate solution was, of course, removed from the cell by thorough washing with distilled water.

At 20° the pH of the phosphate solutions standardized in this way against 0.1 N HCl (assuming pH 1.085) differ according to our experience, by about 0.01 pH from the values assigned to the mixtures by Sørensen. It is felt that this difference is a satisfactory agreement with Sørensen's values.

*Platinum Electrodes.*—Platinum electrodes were plated from platinum chloride solution containing 1 to 2 per cent HCl. Lead acetate was *not* used. After a satisfactory coating was obtained, the electrodes were placed in strong dichromate sulfuric acid cleaning solution and heated over the water bath. The electrodes were then washed and allowed to stand in distilled water over night. This technique was proposed by J. C. Baker and L. L. Van Slyke (1918). Electrodes prepared in this manner may ordinarily be used for a long while, but when used with plasma the only safe procedure is to use fresh electrodes each day or four to six determinations on any one day. Something happens to the electrode used in such protein solutions, probably fibrin precipi-

tation, which makes them unreliable for further use in protein solutions. Electrodes, which will not give consistent consecutive or duplicate results with plasma, will appear perfect when rechecked against standard phosphates. Used again with plasma, no satisfactory reading can be obtained.

This undeterminable error which cannot be detected by the use of standard solutions can be recognized by the disagreement between duplicate measurements or between successive refills. For this reason plasma determinations were always duplicated simultaneously in two separate electrode vessels. The platinum electrodes after use with plasma may be cleaned with cleaning mixture, as outlined, without recoating. Our experience has been that platinum electrodes coated with platinum black are more satisfactory than those coated with palladium.

*Thermostat.*—The 38°C. measurements were carried out in a gas chain mounted in an incubator equipped with 150 watt nitrogen lamps for heating. The temperature was controlled with a De Khotinsky regulator and the air was stirred vigorously by means of a small fan. The temperature was read from a thermometer placed along side the Clark vessel. 10 minutes were required before the temperature of the solution agreed with that of the bath. This was determined by inserting a thermometer into the Clark cell in the manner described elsewhere (Cullen, 1922). Determinations at 20° were carried out in a constant temperature room.

*Corrections.*—The readings, made with a Leeds and Northrup potentiometer and wall galvanometer, were read to 0.2 millivolts and were then corrected for barometric pressure, vapor pressure, and CO<sub>2</sub> tension to 1 atmosphere of dry hydrogen at the temperature of the determination. No corrections were made for liquid junction potentials.

I am indebted to my assistant Mr. Julius Sendroy for his interest and cooperation during the development of this method.

#### SUMMARY.

1. A method for the colorimetric determination of the reaction of the blood is described. It is based upon the experimentally determined factor required to convert the colorimetric

reading of diluted plasma or serum at room temperature to the actual reaction of the plasma undiluted at 38° as determined electrometrically. This factor is somewhat different for plasma and for serum and for bloods of different species, but appears to have a constant value for each system.

2. A convenient and accurate technique for electrometric pH measurements at definite CO<sub>2</sub> tensions is outlined.

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# A COMPARISON OF COLORIMETRIC AND ELECTROMETRIC DETERMINATIONS OF HYDROGEN ION CONCENTRATIONS IN SOLUTIONS CONTAINING CARBON DIOXIDE.

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In connection with the development of a colorimetric method for measuring the reaction of the blood it became important to determine the precision of the colorimetric method as compared with the electrometric method when used with solutions containing carbon dioxide.

Experiments were therefore carried out on solutions of sodium bicarbonate which had been equilibrated with known tensions of carbon dioxide and in a similar manner on solutions of sodium bicarbonate plus sodium phosphate. The colorimetric readings were made under paraffin oil in the manner described in the preceding paper,<sup>1</sup> except that the solutions were not diluted. Each determination reported here is the average of duplicate readings by either two or three observers. Phenol red was used in preference to neutral red because of the marked tendency of neutral red to precipitate out of solution. Sørensen's phosphate standards<sup>2</sup> were prepared at intervals of 0.05 pH and the readings were made to 0.01 pH.

The equilibrations of the carbonate and carbonate-phosphate solutions with CO<sub>2</sub> were carried out in 800 cc. tonometers. The CO<sub>2</sub> tensions were prepared by running the calculated amounts of CO<sub>2</sub> from a burette into partially evacuated tonometers. The solutions were equilibrated in two or three successive tonometers and were then transferred, without exposure to air, to sampling tubes filled with mercury. (The details of the technique will be

<sup>1</sup> Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

<sup>2</sup> Sørensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.



described in a paper appearing in a later number of this Journal by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke.) Samples were then removed for determination. In most experi-

TABLE I.  
*Experiment 14 A.*

Solution used: 0.067 M phosphate (pH 7.4) and  
0.015 M  $\text{Na}_2\text{CO}_3$  mixed in equal parts.  
 $\text{CO}_2$  tension (as saturated).....28.2 mm.  
 Initial  $\text{CO}_2$  tension in Clark cell.....28.7 mm.  
 Temperature of saturation.....20°C.  
 No. of saturations.....2  
 "e" of calomel electrode.....0.2493 volts.

CO <sub>2</sub> tension.	Hydrogen electrode.	E. M. F.		Tem- perature.	Barom- eter.	E. M. F. corrected to 1 atmos- phere of dry H <sub>2</sub> .	pH <sub>20</sub> °	
		1st filling.	2nd filling.				Electro- metric.	Colori- metric.
<i>mm. Hg</i>		<i>volts</i>	<i>volts</i>	<i>°C.</i>	<i>mm. Hg</i>	<i>volts</i>	<i>pH</i>	<i>pH</i>
28.7	A	0.6716	0.6712	20	761	0.6720	7.28	7.28
28.7	B	0.6712	0.6707	20	761	0.6715	7.27	7.27

TABLE II.  
*Experiment 16 A.*

Solution used: 0.067 M phosphate solution (pH 7.35) and  
0.015 M  $\text{Na}_2\text{CO}_3$  mixed in equal parts.  
 $\text{CO}_2$  tension (as saturated).....28.6 mm.  
 Initial  $\text{CO}_2$  tension in Clark cell.....28.7 mm.  
 Temperature of saturation.....20°C.  
 No. of saturations.....2  
 "e" of calomel electrode.....0.2493 volts.

CO <sub>2</sub> tension.	Hydrogen electrode.	E. M. F.		Tem- perature.	Barom- eter.	E. M. F. corrected to 1 atmos- phere of dry H <sub>2</sub> .	pH <sub>20</sub> °	
		1st filling.	2nd filling.				Electro- metric.	Colori- metric.
<i>mm. Hg</i>		<i>volts</i>	<i>volts</i>	<i>°C.</i>	<i>mm. Hg</i>	<i>volts</i>	<i>pH</i>	<i>pH</i>
28.7	A	0.6710	0.6707	20	761	0.6718	7.27	7.27
28.7	B	0.6709	0.6710	20	761	0.6718	7.27	7.27

ments equilibrations were carried out in a 20° water bath. The analyses were made in a 20° constant temperature room. The electrometric determinations were carried out with the same technique and precautions described in the preceding paper.<sup>1</sup> The system was standardized for each experiment with the same

TABLE III.

*Experiment 18 A.\**

Solution used:  $0.10\text{ M Na}_2\text{HPO}_4$   
 $0.03\text{ M Na}_2\text{CO}_3$  } mixed in equal parts.

$\text{CO}_2$  tension (as saturated) ..... a ..... b ..... c  
 at  $38^\circ$  ..... 80.8 mm. 46.7 mm. 27.7 mm.  
 Initial  $\text{CO}_2$  tension in Clark cell at  $20^\circ$  ..... 49.2 mm. 29.4 mm. 17.5 mm.  
 Temperature of saturation .....  $38^\circ\text{C}$ .  
 No. of saturations ..... 2  
 "e" of calomel electrode at  $20^\circ$  ..... 0.2493 volts.

CO <sub>2</sub> tension.	H <sub>2</sub> elec- trode.	E. M. F.		Tem- pera- ture.	Barom- eter.	E. M. F. corrected to 1 atmos- phere of dry H <sub>2</sub> .	pH <sub>20°</sub>	
		1st filling.	2nd filling.				Electro- metric.	Colori- metric.
<i>mm. Hg</i>		<i>volts</i>	<i>volts</i>	<i>°C.</i>	<i>mm. Hg</i>	<i>volts</i>	<i>pH</i>	<i>pH</i>
49.2	B	0.6734	0.6747	20	756	0.6759	7.34	7.35
29.4	A	0.6854	0.6870	20	756	0.6879	7.55	7.56
29.4	B	0.6873	0.6874	20	756	0.6883	7.56	7.56
17.5	A	0.6988	0.6983	20	756	0.6990	7.74	7.74
17.5	B	0.6974	0.6984	20	756	0.6991	7.74	7.74

\* In this experiment the  $\text{CO}_2$  tensions used in the hydrogen-carbon dioxide mixture for the  $20^\circ$  electrometric determination are calculated to give the same  $\text{H}_2\text{CO}_3$  concentration in the solution at  $20^\circ$  as existed in the solution as equilibrated at  $38^\circ$ .

TABLE IV.

*Experiment 24 A.*

Solution used:  $0.03\text{ M Na}_2\text{CO}_3$  converted to  $0.06\text{ M NaHCO}_3$  with  $\text{CO}_2$ .  
 $\text{CO}_2$  tension (as saturated) ..... 40 mm.  
 Initial  $\text{CO}_2$  tension in Clark cell ..... 40 mm.  
 Temperature of saturation .....  $20^\circ\text{C}$ .  
 No. of saturations ..... 3  
 "e" of calomel electrode ..... 0.2486 volts.

CO <sub>2</sub> tension.	Hydrogen electrode.	E. M. F.		Tem- pera- ture.	Barom- eter.	E. M. F. corrected to 1 atmos- phere of dry H <sub>2</sub> .	pH <sub>20°</sub>	
		1st filling.	2nd filling.				Electro- metric.	Colori- metric.
<i>mm. Hg</i>		<i>volts</i>	<i>volts</i>	<i>°C.</i>	<i>mm. Hg</i>	<i>volts</i>	<i>pH</i>	<i>pH</i>
40	A	0.6962	0.6960	20	753	0.6971	7.72	7.70
40	B	0.6951	0.6957	20	753	0.6968	7.71	7.70

phosphate solution of pH 7.4 used as the colorimetric standard. The "c"  $\left( \text{pH} = \frac{\text{E. M. F.} - \text{"e"}}{0.0001984 T} \right)$  thus obtained was used in calculating the pH of the  $\text{CO}_2$ -containing solution. This procedure establishes on phosphate solutions, free from  $\text{CO}_2$ , an identity of pH value for the two methods. The pH values reported are based on the second (refilled) reading, corrected for barometric pressure,  $\text{CO}_2$  tension, and vapor pressure to 1 atmosphere of hydrogen.

The results of four experiments are shown in Tables I to IV.

It is evident from these results that, when both colorimetric and gasometric measurements of hydrogen ion concentration are carried out by the technique described, there is complete agreement between the two methods in both carbonate and phosphate-carbonate solutions.

It has recently been reported by Evans<sup>3</sup> that a discrepancy exists between colorimetric and electrometric determinations of the hydrogen ion concentration of fluids containing carbon dioxide. He concluded that the values determined by the colorimetric method were correct and that the electrometric determinations were consistently 0.2 pH too acid, and suggested the use of the correction + 0.2 pH in electrometric determinations. In view of the fact that, with the technique described, we obtain satisfactory agreement between the two methods we feel that the electrometric method must continue to be regarded as the standard.

#### SUMMARY.

The colorimetric and electrometric methods for determining the hydrogen ion concentration of solutions containing carbon dioxide agree when carried out with rigorous precautions to prevent loss of  $\text{CO}_2$ .

<sup>3</sup> Evans, C. L., *J. Physiol.*, 1921, liv, 353.

# A MODIFICATION OF THE CLARK HYDROGEN ELECTRODE VESSEL TO PERMIT ACCURATE TEMPERATURE CONTROL.

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The hydrogen electrode vessel described by Clark<sup>1</sup> is generally accepted as the most convenient type for use with protein solutions and with biological fluids. It is especially suited for use with solutions containing CO<sub>2</sub>, and in the 2 cc. form used in this laboratory has proved itself entirely satisfactory for use with blood plasma. Clark recommends that the cell be set up in a constant temperature air bath.

We have observed that in running determinations at 38°, 10 to 15 minutes elapse before solutions introduced at room temperature reach the temperature of the bath. When, as rather easily happens, the outside of the cell is wet, it cannot, of course, attain air temperature until the film of water has evaporated, and the time required becomes longer. Cooling by evaporation would disturb temperature equilibrium even if all materials were at thermostat temperature at the start. There is then a definite possibility of error from trusting to judgment in estimating the time required to attain temperature equilibrium.

Further, we have observed that it has become a rather general practice in many laboratories to use the Clark cell in the open room and to read the temperature from a thermometer suspended either in the KCl reservoir or in the air beside the cell. That this reading often does not represent the actual temperature of the cell contents is evident from Tables I and II, which represent the temperature reading made during average experiments in a room where temperature control was not possible. Errors of

<sup>1</sup> Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475; The determination of hydrogen ions, Baltimore, 1920, 128.

TABLE I. \*  
*Temperature Record of pH Measurements at Room Temperature.*

Determination.	Time.	Room. Thermom- eter 1.	KCl cup. Thermom- eter 2.	Cell A. Thermom- eter A.	Cell B. Thermom- eter B.
	<i>a. m.</i>				
1	9.57	22.9	22.5	23.0	23.2
2	10.07	23.0	22.7	23.0	23.0
3	10.15	22.8	22.8	22.9	23.0
Opened window.					
4	10.24	21.5	22.7	22.0	22.2
5	10.28	21.0	22.3	21.8	21.8
6	10.36	20.5	21.8	21.1	21.1
7	10.50	20.0	21.0	20.5	20.6
	<i>p. m.</i>				
	12.40				
Solution at 20° added.					
8	12.45	22.2		20.4	20.5
9	12.55	22.0		21.0	21.0

TABLE II. \*  
*Temperature Record of pH Measurements in Thermostat Set for 37°C.*

Determination.	Time.	Thermom- eter 3.	Thermom- eter 4.	Cell C. Thermom- eter C.	Cell D. Thermom- eter D.
	<i>p. m.</i>				
10	2.46	37.2	37.5	36.0	35.0
11	2.52	37.2	37.5	37.0	37.1
Opened door 1 minute to make contact.					
12	3.54	36.2	37.5	37.0	37.0
13	3.60	37.2	37.5	37.0	37.0
4.00 Put in solution at 22°C.					
14	4.08	37.0	37.5	36.0	36.0
15	4.13	37.2	37.5	37.0	37.0

\* Readings were made with Thermometers 1 and 3 hanging between the two electrode vessels about 1½ inches from each and at the same level. Thermometer 2 was inserted in the KCl cup along side of the connection of the calomel cell and Thermometer 4 in the thermostat was at the back over the lamps. Thermometers A, B, C, and D were inserted in the electrode vessels in the manner described in the text.

0.01 to 0.03 pH would result from the use of the temperature as read from the thermometer suspended between the cells.

The writer has found that errors from these sources may be advantageously obviated by inserting a thermometer into the solution. For this purpose an extra opening in the Clark cell is prepared and a short calibrated thermometer introduced as

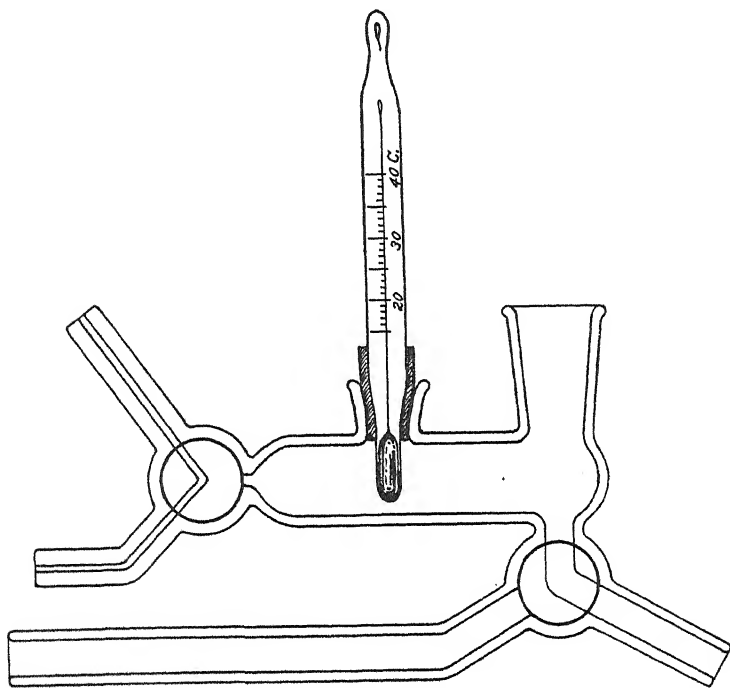


FIG. 1.

shown in Fig. 1. The thermometer is carried in pure rubber tubing but could, of course, be ground in.

The construction is evident from the figure, but with the 2 cc. cells which we use care must be taken to make the thermometer bulb so small that it does not obstruct the flow of solution during the rocking of the electrode. The bulb from the shoulder to the tip must be shorter than the inside diameter of the tube, else it is difficult to insert the thermometer without dead space.

For the small cells we use, we specify that the opening to receive the thermometer be exactly round, 8.5 mm. diameter at the top, and 7.5 mm. at the bottom. The opening is about 5 mm. high. The thermometer must be 5 mm. in diameter and about 48 mm. long over all with mercury bulb 8 mm. long tapering straight from the shoulder to 1 to 2 mm. at the tip. There must be no constriction or bulge at the shoulder.<sup>2</sup>

It is felt that this modification of the Clark cell facilitates its use even in a carefully controlled thermostat and markedly increases accuracy when it is used in the ordinary room.

<sup>2</sup> The cells and thermometer were made for us by Wm. Weigand, 141 Lexington Ave., New York.

# ON THE MEASUREMENT OF BUFFER VALUES AND ON THE RELATIONSHIP OF BUFFER VALUE TO THE DIS- SOCIATION CONSTANT OF THE BUFFER AND THE CONCENTRATION AND REACTION OF THE BUFFER SOLUTION.\*

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## *The Nature and Mode of Action of Buffers.*

In his chapter on buffer action Clark (1920) has traced the development of the knowledge of buffers,<sup>1</sup> which we owe mainly

\* The present paper constitutes the theoretical basis for experimental work, the results of which will follow.

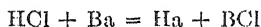
<sup>1</sup> Clark (1920), p. 30.



to the work of L. J. Henderson (1908), and of Sørensen (1912), and has summarized the resultant conception in the statement: "By buffer action we mean the ability of a solution to resist change in pH through the addition or loss of alkali or acid." Reduced to the form of a definition this conception may be expressed as follows: *Buffers are substances which by their presence in solution increase the amount of acid or alkali that must be added to cause unit change in pH.* As will appear later, change in the logarithmic pH unit affords a more convenient measure of buffer effect than change in  $[H^+]$ .

The most efficient buffers, at reactions within the usual range of biological significance, are mixtures of weak acids or weak bases and their salts. Their buffer effect is due to the relatively slight extent to which they undergo electrolytic dissociation, as compared with the almost completely dissociated strong acids and bases.

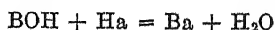
If we add a strong acid, HCl for example, to a mixture of the weak buffer acid, Ha, and its alkali salt, Ba, reaction occurs with approximate completeness from left to right according to the equation



(To represent the weak buffer acid the formula Ha is used to distinguish it from HA, which will be used to indicate a strong, approximately entirely dissociated acid.) In this reaction the strong acid combines with an equivalent of buffer salt and sets free an equivalent of weak buffer acid, Ha. The latter, instead of dissociating like HCl almost entirely into hydrions and anions, dissociates only slightly. Hence the increase in hydrion concentration, and the change in pH, is only a fraction of that which would have been caused by adding the same amount of HCl to an unbuffered solution.

The amount of change that does occur depends on the extent to which the buffer acid set free dissociates according to the equation  $Ha = H^+ + a'$ . If 1 per cent of Ha dissociates into  $H^+$  and  $a'$ , the  $H^+$  increase will be approximately 0.01 of that which would have been caused by adding the HCl to an unbuffered solution.

Similarly, if we add a strong base, BOH, such as KOH or NaOH, to the buffer solution, an equivalent of buffer acid in the Ba, Ha mixture is neutralized according to the equation



The decrease in  $H^+$  concentration due to neutralization of a given amount of  $Ha$  is again dependent on the extent to which the  $Ha$  had been dissociated, since only the  $H^+$  ions owing their presence to that dissociation have disappeared.

It is evident from the above that the  $Ba$  of the buffer mixture reacts when acid is added, and the  $Ha$  when alkali is added, and therefore that both  $Ba$  and  $Ha$  are necessary if the buffer mixture is to offer resistance to reaction change by addition of either acid or alkali. As will be seen later, if ability to minimize the proportion by which any given  $H^+$  concentration is changed is taken as a measure of buffer action, a buffer mixture has its maximum efficiency when  $Ha = Ba$ , half the buffer being free, half in the form of its salt.

When the buffer mixture is composed of a weak base,  $bOH$ , and its salt,  $bA$ , it can be shown similarly that the amount of change in  $H^+$  or  $OII'$  concentration caused by addition of acid or alkali depends on the extent to which  $bOH$  dissociates into  $b^+$  and  $OH'$  ions.

Since weak acids and bases obey in their dissociation the simple law of mass action, and since their buffer action is dependent on the extent of their dissociation, it follows that the quantitative relationships governing buffer action are capable of formulation from the mass law.

By means of such formulation, L. J. Henderson (1908) has shown that buffer acids most efficient in maintaining a neutral reaction of  $[H^+] = [OH'] = 10^{-7}$  are such as have dissociation constants most nearly equal to  $10^{-7}$ . Clark (1920) has pointed out<sup>2</sup> "that it is only within a certain zone of  $\log \frac{1}{[H^+]}$  that a mixture of an acid with its salt produces a stabilized hydrogen ion concentration or pH." The writer has recently indicated (1921, *a*) a mode for the mathematical proof that in general, if buffer efficiency be considered as ability to minimize change in pH, that is, proportional change in  $[H^+]$ , any buffer salt of a weak acid is most efficient when  $[Ha]$  and  $[Ba]$  are equal, under which conditions  $pH = pK'_a$ .<sup>3</sup>

<sup>2</sup> Clark (1920), p. 19.

<sup>3</sup>  $pK'_a$  is an expression introduced by Hasselbalch (1917) to indicate the negative logarithm of  $K'_a$ , the value of  $K'_a$  being  $\frac{K_a}{\gamma}$  where  $K_a$  is the dis-

*Unit for Measurement of Buffer Values.*

Thus far, however, there has appeared in the literature no satisfactory mode of expressing, over the zone of pH at which buffers act, the quantitative relationships of buffer effect to the dissociation constant of the buffer and the reaction: in fact there has been no unit for the numerical expression of buffer effect. It is the purpose of the present paper by means of such a unit to attain quantitative measurement and expression of buffer effects, and to derive from the mass law the above mentioned relationships.

The unit adopted is the differential ratio  $\frac{dB}{dpH}$ , expressing the relationship between the increment (in gram equivalents per liter) of strong base B added to a buffer solution and the resultant increment in pH. Increment of strong acid is equivalent to a negative increment of base, or  $-dB$ . In these terms *a solution has a buffer value of 1 when a liter will take up 1 gram equivalent of strong acid or alkali per unit change in pH.*

If base is added to a solution, pH is increased, so that both  $dB$  and  $dpH$  are positive. If acid is added both  $dB$  and  $dpH$  are negative. The ratio  $\frac{dB}{dpH}$  is, therefore, always a positive numerical value. If one solution has twice the buffer value of a second solution, twice as much base or acid is required to change the pH by a given small amount, for example, 0.1, and therefore the value  $\frac{dB}{dpH}$  is twice as great in the first solution as in the second. For convenience we shall use at times the letter  $\beta$  to indicate the ratio  $\frac{dB}{dpH}$ .

The significance of the ratio  $\frac{dB}{dpH}$  as a measure of buffer effect is illustrated by Fig. 1. In place of  $dB$  and  $dpH$ , infinitesimal

sociation constant of the buffer acid  $[Ha]$ ,  $\gamma$  the degree of dissociation of its salt  $[Ba]$  into  $[B^+]$  and  $[a^-]$  ions. Hasselbalch actually used the form  $K_1$ . We have altered it to  $K'$  in order to facilitate differentiating between  $K_a$  and  $K'_b$ , derived from the acid and basic dissociation constants customarily designated as  $K_a$  and  $K_b$  respectively.

increments, we there use measurable increments  $\Delta B$  and  $\Delta pH$ , which, if not too great, serve our purpose nearly as well. In order to increase the pH of Solution 1 from 3 to 4, 0.1 gram mole-

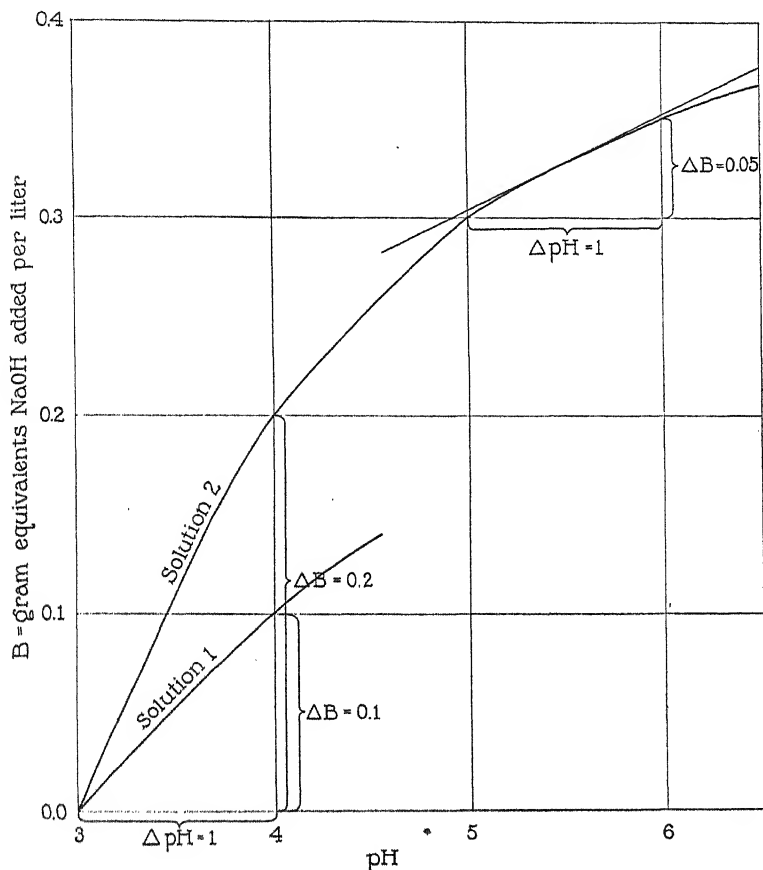


FIG. 1.

cule of NaOH per liter is required; therefore  $\Delta B = 0.1$ . Since  $\Delta pH = 1$  (the increase in pH caused by the change from pH 3 to 4), the approximate buffer value of Solution 1 at the mean reaction of pH 3.5 is  $\beta = \frac{\Delta B}{\Delta pH} = \frac{0.1}{1.0} = 0.1$ .

In order to increase the pH of Solution 2 from pH 3 to pH 4, 0.2 gram molecule of NaOH per liter is required; therefore, for  $\Delta\text{pH} = 1$ ,  $\Delta\text{B} = 0.2$ ; and, at mean pH 3.5,  $\beta = \frac{0.2}{1.0} = 0.2$ .

Let us now follow the curve of Solution 2 further along until it covers the range of pH 5 to 6. Because the curve has become less steep, we find that the  $\Delta\text{B}$  accompanying the pH increase from 5 to 6 is only 0.05. Consequently, Solution 2, which at pH 3.5 had a buffer value of  $\frac{\Delta\text{B}}{\Delta\text{pH}} = 0.2$ , has at pH 5.5 only one of  $\frac{\Delta\text{B}}{\Delta\text{pH}} = 0.05$ .

In place of measuring the  $\Delta\text{B}$  and  $\Delta\text{pH}$  values for equal distances on both sides of the pH at which we wish to determine the value of  $\frac{d\text{B}}{d\text{pH}}$  we may draw a tangent to the curve at the indicated pH

and measure the slope of the tangent. Thus, in the case of Solution 2, we may draw a tangent to the curve at pH 5.5. Since the tangent, being a straight line, has the same slope at all points, we may prolong it as much as we wish in order to determine its slope conveniently. For example, we make the measurements between pH 5 and 6.5. At these points the tangent cuts the B lines at 0.303 and 0.376 respectively, the difference being 0.073.

The slope of the tangent, or the value of  $\frac{d\text{B}}{d\text{pH}}$  at pH 5.5, is therefore  $\frac{0.073}{1.500} = 0.0486$ , which approximates the value  $\frac{\Delta\text{B}}{\Delta\text{pH}} = 0.05$  obtained above. The tangent, if accurately drawn, gives the exact  $\frac{d\text{B}}{d\text{pH}}$  value at its point of contact with the curve, and there-

fore has some theoretical advantage over the use of the measurable increments  $\Delta\text{B}$  and  $\Delta\text{pH}$ , which indicate the approximate buffer value at the mean pH over the portion of curve measured.

The curve of Solution 2 illustrates the fact that a given solution may have a different buffer value at different reactions, and that one may speak in exact terms only of its buffer value at a definite pH, or of its average buffer value over a certain pH range. The manner in which the buffer value of a solution containing a single buffer acid and its salt changes with varying pH is shown by Figs. 4 and 9.

Since a solution may have a different buffer value at every pH, rigid accuracy would require that it be measured by the ratio  $\frac{dB}{dpH}$ , of the infinitesimal increments  $dB$  and  $dpH$ . Actually, as may be seen from Fig. 4, increments  $\Delta B$  and  $\Delta pH$  of sufficient size to be measured may be usually employed with fair accuracy.

*The Buffer Value of Water Plus Only Strong Acid or Alkali.*

When strong acid or alkali is added to water the solution displays a certain buffer value. If it did not, each addition of acid or alkali would cause infinite change in pH.

Let us assume that a strong base completely dissociating into the ions  $[B^+]$  and  $[OH']$  is added to water. Then the increment  $dB$ , in base, is equal to the increment  $d[OH']$ , in  $[OH']$ . Consequently for  $\frac{dB}{dpH}$  we may write  $\frac{d[OH']}{dpH}$ .  $pH = -\log [H^+] = -\log \frac{k_w}{[OH']}$   $= \log [OH'] - \log k_w$ , when  $k_w$  is the water constant  $[OH'] \times [H^+]$ . Hence  $dpH = d \log [OH']$ . Therefore

$$(1) \quad \frac{dB}{dpH} = \frac{d[OH']}{d \log [OH']} = \frac{[OH']}{0.4343} = 2.3 [OH']$$

Similarly, if we add to water a strong acid, completely dissociated into  $[H^+]$  ions and anions, such addition constitutes a negative addition of base. The increment in acid is equal to that in  $[H^+]$ , that is,  $-dB = d[H^+]$ .

$$(2) \quad \frac{dB}{dpH} = \frac{-d[H^+]}{-d \log [H^+]} = \frac{[H^+]}{0.4343} = 2.3 [H^+]$$

If we add the two effects, expressed by Equations 1 and 2, respectively, we express the total buffer value of water plus completely dissociated acid or alkali at all pH's, as shown in Equation 3.

$$(3) \quad \frac{dB}{dpH} = 2.3 ([H^+] + [OH'])$$

At any given  $[H^+]$  or  $[OH']$ , for each gram equivalent of hydron or hydroxyl ion present, completely dissociating base or acid must be added at the rate of 2.3 gram equivalents per liter per unit change in pH effected.

The relationships expressed in Equation 3 are shown in Figs. 2 and 3. It is obvious from them that over the reaction range usually significant in animal or plant physiology, pH 3 to 11, the buffer value of completely dissociated acid or alkali is slight, too slight in fact to show graphically on charts of the scale used, but that at both ends of this range it becomes significant.

The actual dissociation of a strong acid, such as HCl, or a strong base, such as NaOH, is only approximately complete at finite dilutions. If we express the fraction of BOH dissociated into  $[B^+]$  and  $[OH']$  by  $\gamma_B$ ,  $[OH'] = \gamma_B [BOH] = \gamma_B B$ ,  $B = \frac{[OH']}{\gamma_B}$ , and  $dB = \frac{d[OH']}{\gamma_B}$ . Similarly  $dB = \frac{-d[H^+]}{\gamma_A}$  if  $\gamma_A$  represents the degree of dissociation of the strong acid. Consequently, if instead of ideal, completely dissociated strong base and acid, our additions to water are of actual, not quite completely dissociated strong base and acid,  $\gamma_B$  and  $\gamma_A$  being a little less than 1, instead of Equation 3, we have the slightly different Equation 4.

$$(4) \quad \frac{dB}{dpH} = 2.3 \left( \frac{[H^+]}{\gamma_A} + \frac{[OH']}{\gamma_B} \right)$$

The fact that this equation is accurate is illustrated by the following example. 0.1 N HCl is, according to the conductivity data of Noyes and Falk, 92.3 per cent dissociated at 20°.  $[H^+] = 0.0923$  N; pH = 1.035. If we increase the HCl to 0.11 N,  $[H^+]$  increases to  $0.0923 \times 1.10 = 0.1015$  (the dissociation does not change significantly), and pH falls to 0.994. From these measured changes,  $\Delta B$  and  $\Delta pH$ , we calculate the approximate  $\beta$  value at the mean pH between 1.035 and 0.994.

$$\beta = \frac{\Delta B}{\Delta pH} = \frac{0.10 - 0.11}{0.994 - 1.035} = \frac{-0.01}{-0.041} = 0.243$$

The mean pH of the range, pH 1.035 to 0.994, is 1.015. The corresponding  $[H^+]$  is 0.0966 ( $[OH']$  is negligible). The buffer value of the solution, at pH 0.994, calculated by Equation 4, with  $\gamma_A = 0.923$ , is

$$\beta = \frac{dB}{dpH} = 2.3 \frac{0.0966}{0.923} = 0.241$$

Neglecting  $\gamma_A$  and calculating by Equation 3 we would obtain

$$\beta = 2.3 \times 0.0966 = 0.222$$

For HCl solutions more dilute than 0.1 N,  $\gamma_A$  approaches more nearly 1, and the difference between results calculated by Equations 3 and 4, respectively, becomes still less than in this example.

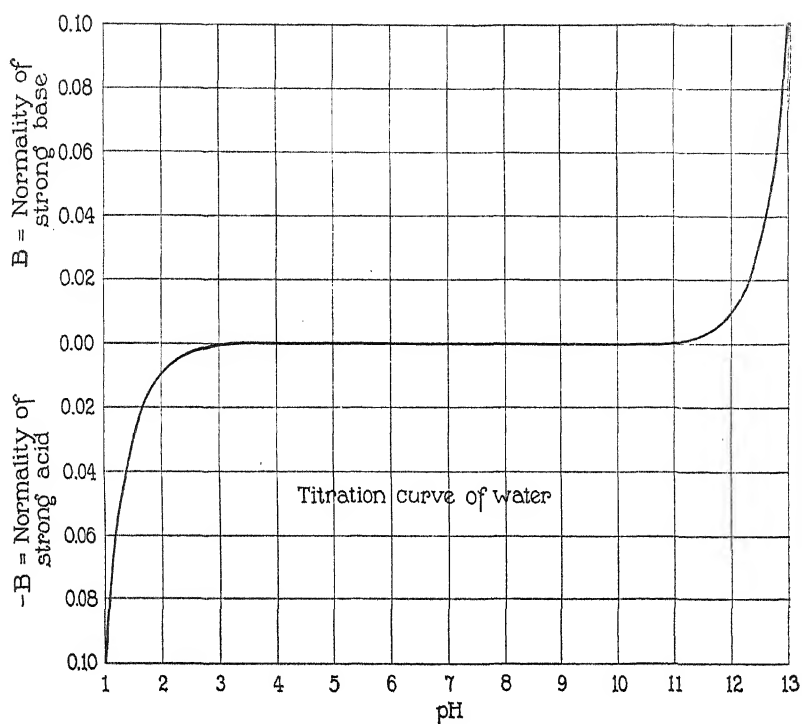


FIG. 2.

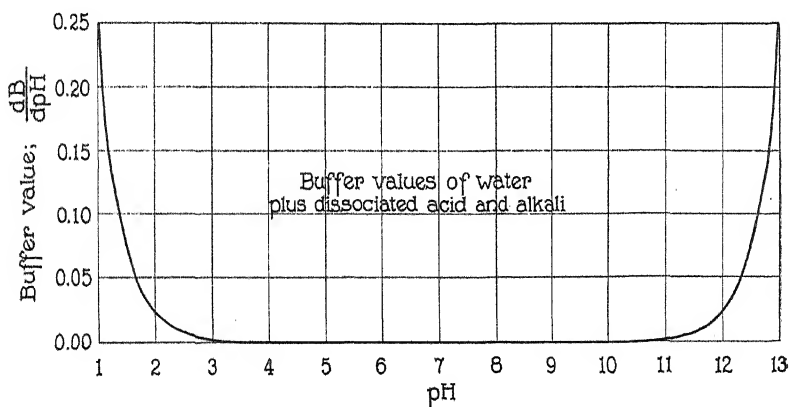


FIG. 3.



The buffer action of a strong, completely dissociated acid or base is different in its mechanism from the buffer action of a weak, slightly dissociated acid or base, such as was discussed in the first section of this paper, and is about to be considered quantitatively in the next section. The buffer action of the weak acid or base involves repression of part of the  $[H^+]$  change that would be caused by addition of acid or alkali to an unbuffered solution. With repression of  $[H^+]$  change there is also, of course, an accompanying repression of the *proportion* by which  $[H^+]$  is altered, and it is this proportional  $[H^+]$  change that is a linear function of the  $\log [H^+]$  or pH change which we have adopted to measure buffer effect.

The fact that changes in  $\log [H^+]$ , and in pH, are linear measures of the proportion by which  $[H^+]$  is changed may be demonstrated as follows. If  $d [H^+]$  is the increment in  $[H^+]$ , the proportion of itself by which  $[H^+]$  is increased is  $\frac{d [H^+]}{[H^+]}$ . If an increment,  $-dB$ , of strong acid is added, with resultant absolute change in  $[H^+]$  of  $d [H^+]$ , we have  $\frac{-d B}{\frac{d [H^+]}{[H^+]}}$  as the ratio between added acid and the proportion by which  $[H^+]$  is increased. But in general,

$$\frac{dx}{\frac{dy}{y}} = \frac{dx}{d \log_e y} = \log_{10} e \frac{dx}{\log_{10} y}$$

$$\text{Hence } \frac{-dB}{\frac{d[H^+]}{[H^+]}} = -\log_{10} e \frac{dB}{\log_{10} [H^+]} = 0.4343 \frac{dB}{dpH}$$

$$dpH = -0.4343 \frac{d[H^+]}{[H^+]}$$

It is obvious from the right-hand member of the last equation that as factors of  $dpH$  we must consider not only  $d[H^+]$ , but also  $[H^+]$ .

The presence of a completely dissociated acid or base, would repress *only* the proportion by which a given further addition would alter the  $[H^+]$ . The absolute  $[H^+]$  change would be practically unaffected by strong acid already present before this addition: only the  $[H^+]$  factor of the  $\frac{d[H^+]}{[H^+]}$  ratio would be affected.

The greater the amount of  $[H^+]$  or  $[OH']$  (or completely dissociated acid or alkali) present, however, the greater the further amount required to be added or removed to change the original to 10-fold or  $\frac{1}{10}$  its own value, and thereby cause unit change in pH. And this further amount is our measure of buffer value. So, if we adopt pH change as our unit of reaction shift, we must assign a definite buffer effect to the mere mass of  $[H^+]$  or  $[OH']$  ions present, which, by its inertia, so to speak, represses pH change. This effect we may term *the buffer effect of the dissociated base or acid, or of the  $[H^+]$  or  $[OH']$  concentration*.

In a sense, therefore, the adoption of  $\frac{dB}{dpH}$  as the unit of buffer value has broadened the conception of buffer action outlined in the introduction. Buffer actions have there been discussed as processes repressing  $[H^+]$  changes by replacement of the added, more or less strongly dissociated acids or bases with the weak, undissociated acids or bases of the buffers. To this action we must add the "inertia" buffer effect peculiar to dissociated acids and bases, which repress the proportional, though not the absolute,  $[H^+]$  change, and which attain importance when  $[H^+]$  or  $[OH']$  exceeds about  $10^{-3}$  (when pH is less than 3 or greater than 11). As will appear later (Equations 17 and 35) the total buffer value of a solution is represented by the sum of the buffer effects due, respectively, to dissociated and to undissociated acids or bases present. The two effects may both be important in a buffer forming a very acid solution, such as  $H_3PO_4$ , or forming a highly alkaline one, such as  $Na_3PO_4$ .

*The Buffer Value of a Solution of a Weak Monovalent Acid and Its Salt.*

*Fundamental Mass Law Equations.*—The fundamental equation derived from the mass action law of Guldberg and Waage as applied by Arrhenius to the dissociation of electrolytes, and shown by Ostwald (1888) to hold for dilute solutions of weak acids is

$$(5) \quad K_a = \frac{[H^+] \times [a']}{[Ha]}$$

where  $[Ha]$  is the undissociated portion of the acid,  $[H^+]$  the hydron concentration,  $[a']$  the concentration of anions formed by

dissociation of the acid, and  $K_a$  the dissociation constant of the acid.

This equation has been applied in two forms. It was used by Ostwald (1888) for pure solutions of weak acids in the form

$$(6) \quad K_a = \frac{[H^+]^2}{[Ha]}$$

The theoretical accuracy of Equation 6 for such solutions follows from the fact that both hydrions and anions must arise in equal numbers from the dissociation of  $[Ha]$ , when the latter is their sole significant source.

For solutions containing not only the free acid, but also its salt,  $[Ba]$ , Equation 5 was converted by L. J. Henderson (1908, 1909) into the form

$$(7) \quad K_a = \frac{[H^+] \gamma_s [Ba]}{[Ha]}$$

$[Ha]$  = concentration of free undissociated acid.

$[Ba]$  = total concentration of salt, dissociated or undissociated.

$\gamma_s$  = fraction of  $[Ba]$  dissociated into  $[B^+]$  and  $[a']$ .

$\gamma_s[Ba]$  = concentration of  $a'$  formed by dissociation of  $[Ba]$ .

The salt is usually 80 per cent or more dissociated ( $\gamma_s = 0.8$  to 1.0) while the free acid is dissociated to a relatively negligible extent into  $[H^+]$  and  $[a']$ . The slight amount of  $[a']$ , equal to the  $[H^+]$ , arising from dissociation of the free acid is, at reactions covering a considerable zone on both sides of neutrality, negligible in comparison to the amount formed from the salt. Consequently, over a pH range of about  $7 \pm 3$  or 4,  $\gamma_s[Ba]$  can be inserted, with a negligible error in place of  $[a']$  in Equation 5. (A certain inconsistency in symbols occurs through the use of  $[Ha]$  to represent only the undissociated part of the acid, while  $[Ba]$  represents the total salt, dissociated and undissociated. It has seemed preferable, however, to retain  $[Ba]$  with this significance, partly because it has been used in this manner in the literature since Henderson introduced his equation, partly because it has the advantage of being a self-explanatory symbol.)

Hasselbalch (1917) expressed Henderson's equation in the logarithmic form

$$(8) \quad pH = pK'_a + \log \frac{[Ba]}{[Ha]}$$

$pK'_a$  being the negative logarithm of  $K'_a$ , while  $K'_a = \frac{K_a}{\gamma_s}$ .

Equation 7, and its equivalent, Equation 8, hold for all  $[H^+]$  values that are found in body fluids and excretions (except perhaps gastric juice) since the conditions are fulfilled that  $[H^+]$  and  $[OH']$  are both small (in the neighborhood of  $10^{-7}$  in the internal fluids, and not exceeding  $10^{-5}$  in urine), and the concentration of anions formed by dissociation of the buffer salts present is sufficient to make  $\gamma_s [Ba]$  the only factor that requires consideration in estimating  $[a']$ . Equations 7 and 8 are therefore accurate for the physiological range for which Henderson used them, and may in fact be used over a still wider reaction range. The limitations of this range will be considered later.

*Differentiation of Henderson's Equation in Order to Calculate the Buffer Values of Weak Acids.*—In order to obtain the buffer value,  $\frac{dB}{dpH}$ , in terms of the buffer concentration and reaction, we convert Equation 7 into the form

$$(9) \quad K_a = \frac{[H^+] \gamma_s B}{C - B}$$

$C$  = total molecular concentration of buffer acid before addition of base.

$B$  = gram equivalents of strong base added.

$C - B = [HA]$

Within the range of validity of Henderson's equation, all the BOH added takes the form of the buffer salt,  $[Ba]$ , the base remaining free as BOH being negligible. Therefore, we may substitute  $B$  for  $[Ba]$  in Equation 7. Furthermore, since all save a negligible portion of the buffer acid is in the form either of free undissociated acid,  $[Ha]$ , (the negligible residue is dissociated into  $[H^+]$  and  $[a']$ ), or salt,  $[Ba]$ , we may substitute  $C - B$  for  $[Ha]$ . With these two substitutions Equation 7 is changed into Equation 9.

Solving Equation 9 for  $B$  we have

$$(10) \quad B = \frac{K_a C}{K_a + \gamma_s [H^+]} = \frac{K'_a C}{K'_a + [H^+]} \quad (\text{where } K_a \gamma_s = K'_a)$$

$$\frac{dB}{dpH} = - \frac{dB}{d \log [H^+]} = - \frac{[H^+]}{0.4343} \times \frac{dB}{d[H^+]} = - 2.3 [H^+] \frac{dB}{d[H^+]}$$

Differentiating the first form of Equation 10,  $B = \frac{K_a C}{K_a + \gamma_s [H^+]}$ , we have

$$\frac{dB}{d[H^+]} = - \frac{K_a \gamma_s C}{(K_a + \gamma_s [H^+])^2}$$

From which we obtain, by multiplying  $\frac{dB}{d[H^+]}$  by  $-2.3 [H^+]$ ,

(11)	$\frac{dB}{dpH} = \frac{2.3 K_a \gamma_s C [H^+]}{(K_a + \gamma_s [H^+])^2}$	$\left. \begin{array}{l} \text{Equivalent} \\ \text{expressions} \\ \text{for the buffer} \\ \text{value } \frac{dB}{dpH}, \\ \text{or } \beta, \\ \text{for weak acids.} \end{array} \right\}$
(12)	$= \frac{2.3 K'_a C [H^+]}{(K'_a + [H^+])^2}$	
(13)	$= \frac{2.3 [Ba] [H^+]}{(K'_a + [H^+])}$	
(14)	$= \frac{2.3 [Ba] [Ha]}{C}$	

Equation 12 is derived from Equation 11 by substituting  $K'_a \gamma_s$  for its equivalent  $K_a$ , or by differentiation of the second form of Equation 10, in which this substitution is already made. Equation 13 is derived from Equation 12 by substituting  $[Ba]$  for

$\frac{K'_a C}{K'_a + [H^+]}$ . (Henderson's equation may be written  $K'_a (C - [Ba]) = [H^+] [Ba]$ , whence  $[Ba] = \frac{K'_a C}{K'_a + [H^+]}$ ). Equation 14

is derived from Equation 13 by substituting  $[Ha]$  for  $\frac{[H^+] C}{K'_a + [H^+]}$ . (If Henderson's equation is written  $K'_a [Ha] = [H^+] (C - [Ha])$  it yields this value for  $[Ha]$ ).

We have tested the approximate accuracy of Equation 14 (and therefore of its equivalents, Equations 11, 12, and 13) against experimental results by applying it to Clark and Lubs' data for  $KH_2PO_4 - KHPO_4$  mixtures (1916), the pH values being electrometrically determined by Clark for varying  $[Ba]:[Ha]$ , or  $K (KHPO_4) : H (KH_2PO_4)$ , ratios. Considering the short intervals used ( $\Delta pH = 0.2$ ), the agreement between the theoretically calculated  $\frac{dB}{dpH}$  values and those approximated from the

experimental  $\Delta B$  and  $\Delta pH$  figures is as satisfactory as could be expected from this mode of calculation.

The data of Table I are expressed graphically in Fig. 4, the curve being calculated from Equation 8 with  $pK'_a = 6.85$ . Clark's experimentally determined points are indicated by crosses.

It is evident from Equation 12 that the buffer effect expressed in terms of  $K_a$  and  $[H^+]$  is proportional to the total molecular concentration  $[C]$  of the buffer. We may therefore divide the derivative by  $[C]$ , in order to determine the *molecular buffer value* of a solution, or the buffer value which an  $M$  solution of it would have. This unit may be used for comparison of different buffers. It becomes

$$(15) \quad \frac{dB}{CdpH} = \frac{2.3 K'_a [H^+]}{([H^+] + K'_a)^2} = \beta_m$$

We shall hereafter refer for convenience to the absolute buffer value as  $\beta$ , the molecular buffer value as  $\beta_m$ . The relationship between the two is

$$(16) \quad \frac{\beta}{C} = \beta_m$$

The total buffer value of a solution of a weak acid to which both strong acid and alkali are added in amounts not limited to equivalence with the buffer acid, may be expressed as the  $\beta$ , of the buffer plus the  $\beta$  of the free, dissociated acid or alkali. This is expressed by combining Equations 3 and 12 in Equation 17.

$$(17) \quad \beta = 2.3 \left( \frac{K'_a C [H^+]}{(K'_a + [H^+])^2} + [H^+] + [OH'] \right)$$

For acetic acid ( $pK'_a = 4.6$ ) in 0.2 and 0.1  $M$  concentrations, the total buffer value of the solution from pH 1 to 13 is indicated by Fig. 5. Where the buffer effects of the acetate and hydron overlap, the separate  $\beta$  values are indicated by broken lines, the actual total  $\beta$  values of the solution by continuous lines.

The use of  $\frac{\beta}{C}$  as  $\beta_m$  is obviously permissible only where  $[H^+]$  and  $[OH']$  are negligible in comparison with  $\frac{K'_a C [H^+]}{(K'_a + [H^+])^2}$  which is the case, with  $C = 0.1 M$ , between pH values of about 3 and 11.

TABLE I.

Values of  $\frac{\Delta B}{\Delta pH}$ , as Estimated from Values of  $\Delta B$  and  $\Delta pH$  Taken Directly from W. M. Clark's Curves for 0.05 M  $KH_2PO_4$  Plus NaOH, Compared with Values of  $\frac{dB}{dpH}$  Calculated by Equation 14.

Phosphate mixtures from Clark. C = 0.05 M										
pH	Mean pH.	$\Delta pH$	[Ba]	$pK'_a$ *	Mean [Ba].	Mean [Ha].	$\Delta B$	$\frac{\Delta B}{\Delta pH}$	$\frac{dB}{dpH}$ calculated as 46 [Ba] <sup>†</sup> from mean [Ha] and [Ba].	Molecular buffer value $\beta_M$ .
5.8	5.9	0.2	0.0037	6.90	0.0047	0.0453	0.0020	0.0100	0.0098	0.200
6.0	6.1	0.2	0.0057	6.89	0.0062	0.0438	0.0029	0.0145	0.0125	0.290
6.2	6.3	0.2	0.0086	6.88	0.0106	0.0394	0.0040	0.0200	0.0192	0.400
6.4	6.5	0.2	0.0126	6.87	0.0152	0.0348	0.0052	0.0260	0.0245	0.520
6.6	6.7	0.2	0.0178	6.86	0.0207	0.0293	0.0058	0.0290	0.0279	0.580
6.8	6.9	0.2	0.0236	6.85	0.0266	0.0234	0.0060	0.0300	0.0287	0.600
7.0	7.1	0.2	0.0296	6.84	0.0323	0.0177	0.0054	0.0270	0.0263	0.540
7.2	7.3	0.2	0.0350	6.83	0.0372	0.0123	0.0045	0.0225	0.0211	0.450
7.4	7.5	0.2	0.0395	6.83	0.0411	0.0089	0.0033	0.0165	0.0168	0.330
7.6	7.7	0.2	0.0428	6.83	0.0440	0.0060	0.0024	0.0120	0.0121	0.240
7.8	7.9	0.2	0.0452	6.83	0.0450	0.0050	0.0016	0.0080	0.0104	0.160
8.0			0.0468	6.83						0.208

\* Calculated as  $pK'_a = pH - \log \frac{[Ba]}{0.05 - [Ba]}$ .

†  $\frac{2.3}{C} = \frac{2.3}{0.05} = 46$  (Equation 14).

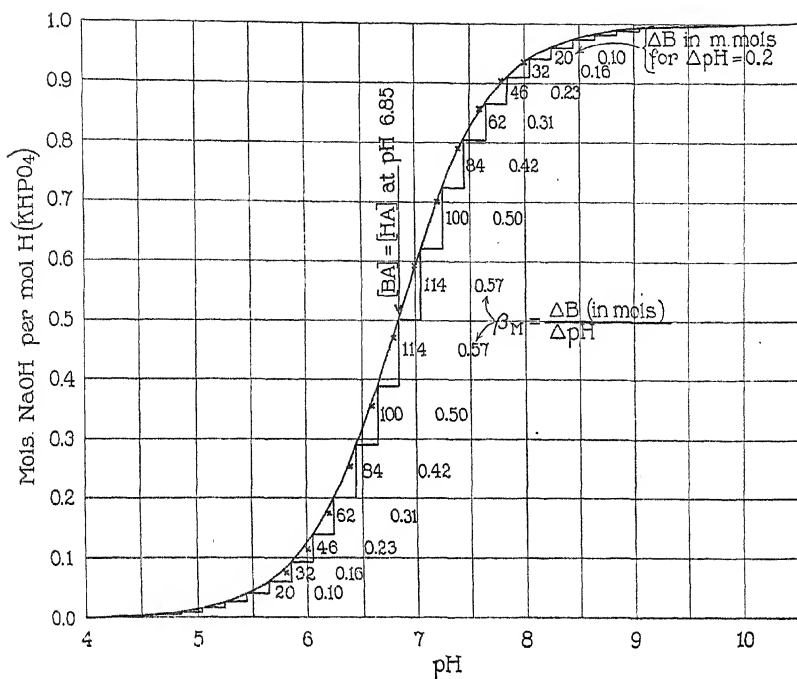


FIG. 4. Titration curve of  $\text{H}(\text{KH}_2\text{PO}_4)$  plus  $\text{NaOH}$ . Curve is calculated from equation,  $\text{pH} = 6.85 + \log \frac{\text{Na}(\text{KH}_2\text{PO}_4)}{\text{H}(\text{KH}_2\text{PO}_4)}$ . Crosses are from experimental data of Clark and Lubs.

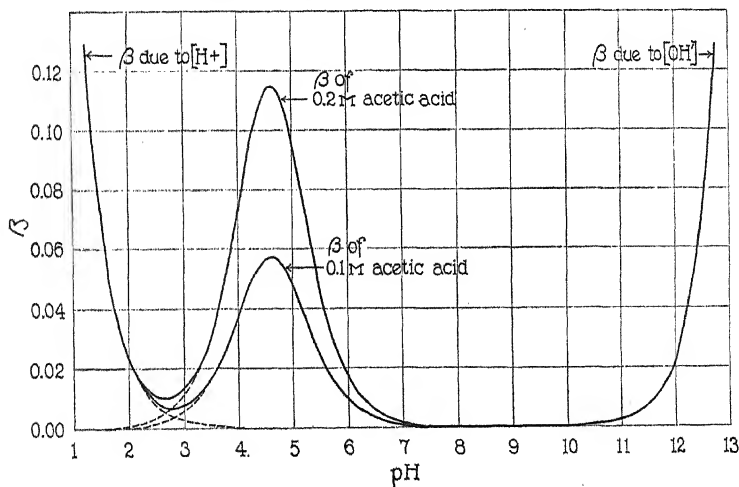


FIG. 5.



*Second Derivative of Henderson's Equation. Point of Maximum Buffer Value.*—To find the conditions under which a mixture of a weak acid and its salt exerts maximum buffer effect, we repeat the differentiation. From Equation 12,  $\beta_m = 2.3 K'_a \frac{[H^+]}{K'_a + [H^+]}$

$$\begin{aligned} \frac{d\beta_m}{d[H^+]} &= 2.3 K'_a \frac{[K'_a]^2 - [H^+]^2}{(K'_a + [H^+])^4} = 2.3 K'_a \frac{K'_a - [H^+]}{(K'_a + [H^+])^3} \\ (18) \quad \frac{d\beta_m}{dpH} &= -2.3 [H^+] \frac{d\beta_m}{d[H^+]} = 2.3 K'_a [H^+] \frac{[H^+] - K'_a}{(K'_a + [H^+])^3} \end{aligned}$$

From Equation 18 it is evident that  $\beta_m$ , the molecular buffer value, has either a maximum or a minimum value when  $[H^+] = K'_a$ , since this is the condition for making  $\frac{d\beta_m}{dpH}$  equal to zero. The fact that maximum, not minimum, value of  $\beta_m$  occurs when  $[H^+] = K'_a$  is obvious from an inspection of the curve of Fig. 4. The occurrence of the maximum at this point was somewhat less rigidly shown by the writer in a former paper (1921, a).

At the maximum point, since  $[H^+] = K'_a$ , we have from Equation 15

$$(19) \quad \beta_m = \frac{2.3 [H^+]^2}{(2[H^+])^2} = \frac{2.3}{4} = 0.575$$

For every buffer this slope indicates a value of  $[Ha] = [Ba]$ , since when  $K'_a = [H^+]$ ,  $[Ha]$  must equal  $[Ba]$ , as is evident when Henderson's equation is put into the form  $[Ba] [H^+] = [Ha] K'_a$ .

The relationships developed lead to the following conclusions, an example of each of which may be obtained from Table I and Fig. 4.

1. All monovalent acid buffers acting within the range of validity of Henderson's equation (with  $pK'_a$  between about 4 and 10) have the same maximum molecular buffer value, *viz.*  $\beta_m = 0.575$ , which they exert when  $[H^+] = K'_a$ , or  $pH = pK'_a$ . (In the case of  $H(KHPO_4)$ , used in Table I,  $pK'_a = 6.85$ .)

2. At this pH,  $[Ba] = [Ha] = \frac{C}{2}$ , that is, half the buffer acid is free, half in the form of alkali salt.

3. If the point of maximum slope be determined in a buffer curve, with increments of added alkali as ordinates, pH values

as abscissæ, one may calculate the molecular concentration of the buffer by Equation 20, which is a rearrangement of Equation 19.

$$(20) \quad C = \frac{\beta}{0.575}$$

*i.e.*, the molecular concentration of the buffer is found by dividing the tangent of the maximum slope of the titration curve by 0.575.

Equation 19 holds even for polybasic acids, provided the dissociation constants of the different acid hydrogen atoms are far enough apart so that, at the pH of its maximum buffer efficiency, each one exerts a buffer effect relatively uninfluenced by the others. If two buffer acids are in equal concentration in the same solution, their  $pK'$  values must be at least 2.5 apart in order that the buffer effect of each may be less than 1 per cent that of the other at the latter's maximum. This is the case with phosphoric acid, of which the three  $pK'$  values are 2.0, 6.8, and 12.0, as seen from Fig. 6. The manner in which polybasic acids may be handled when their buffer effects overlap is discussed later.

It is to be noted that the relationship  $[Ba] = [Ha]$  at a point of maximum buffer value in such a polyvalent acid buffer refers to  $[Ba]$  only as the amount of base replacing the hydrogen of the particular acid group which is acting as a buffer at the existing pH.

4. The pH at the point of maximum slope of the buffer curve indicates the value of  $pK'_a$ .

5. For  $\beta$  values other than the maximum,  $K'_a$  may be calculated by solving Equation 12 for  $K'_a$ . We obtain thus from Equation 12

$$(22) \quad K'_a = [H^+] \frac{C - 0.8686\beta_M \pm \sqrt{(0.8686\beta_M - C)^2 - 0.756\beta_M^2}}{0.8686\beta_M}$$

Of the two  $K'_a$  values obtained by Equation 22, that is correct which is identical with  $[H^+]$  at the point of maximum  $\beta$  value, or the  $\beta_M$  value of 0.575. While ordinarily there is no advantage in using values of  $\beta$  or  $\beta_M$ , and Equation 22, rather than values of  $[Ba]$  and Equation 7 in calculating an acid dissociation constant, yet, there may be occasions in which  $\beta$  is more readily determinable than  $[Ba]$ , and in which, therefore, such an advantage exists.

The calculations indicated by Equation 22 may be performed graphically with the aid of the curve (Fig. 9) expressing values of  $U (U = \frac{\beta_M}{0.575} = 1 \text{ for maximum } \beta_M)$  and of  $\log \frac{[H^+]}{K'_a}$  (which is equal to  $pK'_a - pH$ ) plotted by L. J. Henderson in the note forming part of this paper. By this curve, when  $\beta_M$  is known, the corresponding value of  $pK'_a - pH$ , and hence the value of  $pK'_a$  may be found by inspection. Likewise, when the  $pK'_a$  of an acid buffer is known, the  $\beta$  value at any  $pH$  may be found on the curve.

If instead of taking  $\frac{dB}{dpH}$  as the unit of buffer value we used  $\frac{dB}{d[H^+]}$ , or, to obtain positive values,  $\frac{dA}{d[H^+]}$  where  $A = -B = \text{added strong acid}$ , we would obtain

$$(a) \quad \frac{dA}{d[H^+]} = -\frac{dB}{d[H^+]} = \frac{CK'_a}{(K'_a + [H^+])^2}$$

The molecular buffer value in these terms would be

$$(b) \quad \frac{dA}{Cd[H^+]} = \frac{K'_a}{(K'_a + [H^+])^2}$$

The second derivation is

$$(c) \quad \frac{d^2A}{Cd[H^+]^2} = -\frac{2K'_a}{(K'_a + [H^+])^3}$$

From Equation c it is apparent that there is no point of maximum  $\frac{dA}{d[H^+]}$  value. The second derivative at no point changes through zero from a positive to a negative value or *vice versa*. It is always negative, showing that  $\frac{dB}{d[H^+]}$  decreases continuously as long as  $[H^+]$  has any finite value. For mixtures containing only the free buffer acid and its salt, without additional alkali, minimum  $[H^+]$ , and therefore maximum  $\frac{dA}{d[H^+]}$  values, are obtained when all the acid is bound as salt. At this point  $\frac{dA}{d[H^+]} = \frac{1}{K'_a}$ . Yet experience has led experimenters to decide that weak acids act most efficiently as buffers when they are present about half as free acid,  $[Ha]$ , half as salt,  $[Ba]$  (Clark,<sup>2</sup> 1920). For buffer mixtures containing equal parts of  $[Ha]$  and  $[Ba]$ , the value of  $\frac{dA}{d[H^+]}$  is  $\frac{1}{4K'_a}$  (obtained

by substituting in Equation b  $\frac{K'_a [\text{Ha}]}{[\text{Ba}]}$  for the value of  $[\text{H}^+]$  obtained by solving for  $[\text{H}^+]$  Henderson's equation  $K'_a = \frac{[\text{H}^+] [\text{Ba}]}{[\text{Ha}]}$ . This means that, with half the buffer acid free in each case, half in the form of its salt, the  $\frac{dA}{d[\text{H}^+]}$  values for the three phosphoric acids,  $\text{H}(\text{H}_2\text{PO}_4)$ ,  $\text{H}(\text{HKPO}_4)$ , and  $\text{H}(\text{K}_2\text{PO}_4)$ , would be in round numbers, respectively,  $\frac{1}{4 \times 10^{-2}}$ ,  $\frac{1}{4 \times 10^{-7}}$ , and  $\frac{1}{4 \times 10^{-12}}$ , or 25, 2,500,000, and 250,000,000,000. In the most alkaline of the three solutions, where  $\text{H}(\text{K}_2\text{PO}_4) = \text{K}_3\text{PO}_4$ ,  $\frac{dA}{d[\text{H}^+]}$  is enormous, merely because  $[\text{H}^+]$  is infinitesimal and  $d[\text{H}^+]$  is proportionally reduced. If we based the unit of buffer value on ability to minimize  $[\text{OH}']$  instead of  $[\text{H}^+]$  change, that is, on the value of  $\frac{dB}{d[\text{OH}']}$ , conditions would be reversed, and the buffer value of the alkaline phosphate mixture would be estimated as a billion times greater than that of the acid phosphate. If, to avoid this embarrassment, we used  $\frac{dA}{d[\text{H}^+]}$  for the acid range,  $\frac{dB}{d[\text{OH}']}$  for the alkaline, the acid and alkaline phosphate mixtures would each have similar buffer values, but 100,000 times greater than the neutral  $\text{H}(\text{KHPO}_4) - \text{K}(\text{KHPO}_4)$  mixture. Similar huge ranges of figures are encountered if we use the unit  $1 - \frac{d[\text{H}^+]}{dA}$ .

These anomalies and inconveniences disappear when we use the logarithmic  $\frac{dB}{dpH}$  unit. Each buffer in terms of this unit has the same maximum effect,  $\frac{dB}{dpH} = 0.575$  C, when  $[\text{Ha}] = [\text{Ba}]$ , and each has within the zone where its effect is exerted, the same symmetrical curve of buffer value, the U-curve of Fig. 9.

For expressing reaction changes, Clark and Lubs (1916) have emphasized the advantages of Sørensen's pH unit, due to its avoidance of huge range of numbers, to the elegance with which pH data over wide ranges of reaction may be graphically plotted, to the fact that errors in our methods of determination are proportional to changes in pH rather than in  $[\text{H}^+]$ , and to the fact

that changes in pH rather than in  $[H^+]$  appear to be proportional to changes in physiological effects.

For expressing buffer values the pH unit adds to these advantages that of yielding a uniform and symmetrical  $\beta$  curve for buffers, and a uniform maximum value.

The curve<sup>4</sup> of Van Slyke and Zacharias (1914), showing the rate of action of urease in M/11 phosphate at varying pH, affords an illustration of the parallelism between pH change (or proportional  $[H^+]$  change) and effect on an enzymic activity, and of the relative lack of parallelism between absolute  $[H^+]$  change and effect on activity. In Table II are indicated the pH and  $[H^+]$  changes accompanying the same decrease in rate of enzyme action on both sides of the optimum pH. Approximately the same pH

TABLE II.

	Acid side of optimum.	Alkaline side of optimum.
pH when urease reaction rate = 0.4.....	6.25	8.47
" " " " " = 0.3.....	5.80	8.90
" change.....	-0.45	+0.43
$[H^+]$ when reaction rate = 0.4.....	$5.6 \times 10^{-7}$	$0.034 \times 10^{-7}$
" " " " " = 0.3.....	$15.9 \times 10^{-7}$	$0.012 \times 10^{-7}$
" change.....	$+10.3 \times 10^{-7}$	$-0.022 \times 10^{-7}$
Mean $[H^+]$ .....	$10.7 \times 10^{-7}$	$0.023 \times 10^{-7}$
Proportion by which $[H^+]$ is changed = $\frac{[H^+] \text{ change}}{\text{mean } [H^+]}$ .....	+0.96	-0.96

change, positive on one side, negative on the other, accompanies the same decrease in rate of enzyme action on both the alkaline and acid sides. It follows that the proportions, +0.96 and -0.96, by which  $[H^+]$  is changed are (in this case exactly) equal. But the absolute  $[H^+]$  change on the acid side,  $10.3 \times 10^{-7}$  is about 500 times that ( $0.02 \times 10^{-7}$ ) required on the alkaline side to cause the same decrease in rate of enzyme action. The parallelism between pH change and effect on biological reactions is not, of course, regularly as accurate as happens to be the case in this example. But it appears to be true that rates of enzyme action (and most reactions in the organism appear to be enzymic) as a rule form fairly symmetrical curves when plotted against pH or  $\log [H^+]$ , with some degree of quantitative parallelism on both sides of the optimum between pH change and its effect; while there is no such approach to parallelism between effect and absolute  $[H^+]$  change.

<sup>4</sup> Van Slyke and Zacharias (1914), p. 194.

*General Equation Indicating the Dissociation of a Weak Acid at All Reactions within the Limits of Validity of the Mass Law.*—As stated above Henderson's equation is valid only within a range of reaction which is ordinarily  $7 \pm 3$  or 4, and in consequence the equations expressing the values of  $\beta$  that have been derived above by differentiation of Henderson's equation are subject to the same limitation. In order to express the relationships of  $\beta$  to the hydron concentration of the solution, and the concentration and dissociation constant of the buffer acid, in an equation valid for as great a range of conditions as that limiting the validity of the mass law for dilute acids, we must derive a form of Equation 5 that is general for mixtures of weak acids and their salts, and not limited to the condition that  $[H^+]$  and  $[OH']$  shall be negligible in comparison with C and [Ba]. In order to obtain the desired equation in a form that can be differentiated for calculation of  $\frac{dB}{dpH}$  it is necessary furthermore to express the values of  $[a']$  and [Ha] in terms of C and B.

Let C = total molecular concentration of buffer acid, in form of either free acid or its salt.

B = concentration of strong base BOH added to acid (gram equivalents of base per liter of solution).

[Ba] = concentration of salt Ba.

[Ha] = concentration of undissociated free buffer acid.

$[a']$  = concentration of anion formed by dissociation of the acid Ha and the salt Ba.

$\gamma_s$  = fraction of buffer salt Ba dissociated into  $B^+$  and  $a'$  ions.

$\gamma_B$  = fraction of free strong base BOH dissociated into  $B^+$  and  $OH'$  (BOH is, of course, present only in alkaline solutions such as that of  $Na_2PO_4$ ).

We wish to ascertain general values of  $[A']$  and [Ha] to insert in the basic Equation 5,  $K_a[Ha] = [H^+][a']$ . The value of  $[a']$  may be ascertained by equating the concentrations of all the negative and positive ions, respectively.

$$\begin{aligned}
 (23) \quad [B^+] + [H^+] &= [a'] + [OH'] \\
 [a'] &= [B^+] + [H^+] - [OH'] \\
 &= [B^+] + [H^+] - \frac{K_w}{[H^+]}
 \end{aligned}$$

The terms constituting functions of  $[H^+]$  in the right-hand member we may use as they are.  $[B^+]$ , however, we must obtain in terms of the total base added,  $B$ , and of  $[H^+]$ .

$$(24) \quad [B^+] = \gamma_s [Ba] + \gamma_B [BOH]$$

$$[BOH] = \frac{[OH']}{\gamma_B} = \frac{K_w}{\gamma_B [H^+]}$$

$$[Ba] = B - [BOH]$$

$$(25) \quad = B - \frac{K_w}{\gamma_B [H^+]}$$

Substituting in Equation 24 for  $[BOH]$  its equivalent as given above, and for  $[Ba]$  its equivalent from Equation 25 we have

$$\begin{aligned} [B^+] &= \gamma_s \left( B - \frac{K_w}{\gamma_B [H^+]} \right) + \frac{K_w}{[H^+]} \\ &= \gamma_s B + \left( 1 - \frac{\gamma_s}{\gamma_B} \right) \frac{K_w}{[H^+]} \end{aligned}$$

Substituting the above value for  $[B^+]$  in Equation 23 we have

$$[a'] = \gamma_s B + \left( 1 - \frac{\gamma_s}{\gamma_B} \right) \frac{K_w}{[H^+]} + [H^+] - \frac{K_w}{[H^+]}, \text{ or}$$

$$(26) \quad [a'] = \gamma_s B + [H^+] - \frac{\gamma_s K_w}{\gamma_B [H^+]}$$

The concentration of undissociated free buffer acid,  $[Ha]$ , we find by subtracting from the total buffer concentration,  $C$ , the part, represented by  $[Ba]$ , in the form of salt, and the part, equal to  $[H^+]$ , in the form of free acid dissociated according to the equation  $[Ha] = [H^+] + [a']$

$$[Ha] = C - [Ba] - [H^+]$$

Substituting for  $[Ba]$  its value, as found above in Equation 25, we have

$$(27) \quad [Ha] = C - B + \frac{K_w}{\gamma_B [H^+]} - [H^+]$$

Substituting in our basic Equation 5 the above values for  $[a']$  and  $[Ha]$  gives us

$$(28) \quad K_a = \frac{[H^+] \left( \gamma_s B - \frac{\gamma_s [OH']}{\gamma_B} + [H^+] \right)}{C - B + \frac{[OH']}{\gamma_B} - [H^+]}$$

Substituting  $K'_a \gamma_s$  for  $K_a$ , and dividing both sides of the equation by  $\gamma_s$ , we obtain

$$(29) \quad K'_a = \frac{[H^+] \left( B - \frac{[OH']}{\gamma_B} + \frac{[H^+]}{\gamma_s} \right)}{C - B + \frac{[OH']}{\gamma_B} - [H^+]}$$

With the introduction of but slight error we may simplify Equation 29 by substituting 1 for  $\gamma_B$ , since, in solutions more dilute than 0.01 N, NaOH and KOH approach complete dissociation.

We may also, in the term  $\frac{[H^+]}{\gamma_s}$  substitute 1 for  $\gamma_s$ , since in weak buffer acid-salt solutions of such high acidity that  $[H^+]$  is an important factor in the equation, the ratio  $\frac{[Ha]}{[Ba]}$  is sure to be high, with  $[Ba]$  correspondingly dilute, and  $\gamma_s$  approaching 1 in consequence. Thus simplified Equation 29 becomes

$$(30) \quad K'_a = \frac{[H^+] (B + [H^+] - [OH'])}{C - (B + [H^+] - [OH'])}$$

In solutions of free acids,  $B = 0$  and  $[OH']$  is negligible. Equation 30 then becomes  $K_a = \frac{[H^+]^2}{C - [H^+]}$ , which is equivalent to Ostwald's dilution law (Equation 6) since, with only free acid present,  $C - [H^+] = [Ha]$ . In a solution containing both buffer salts and free acid, and with  $[H^+]$  and  $[OH']$  both insignificant in comparison with  $B$  (solution bordering neutrality), Equation 30 becomes  $K'_a = \frac{[H^+] B}{C - B}$ , which is equivalent to Henderson's equation (Equation 7). Both Ostwald's and Henderson's equations are, therefore, special cases of Equation 30.



In acid and near-neutral solutions ( $\text{pH} < 10$ ),  $[\text{OH}']$  becomes negligible and Equation 30 becomes

$$(31) \quad K'_a = \frac{[\text{H}^+] (B + [\text{H}^+])}{C - (B + [\text{H}^+])}$$

In alkaline and near-neutral solutions ( $\text{pH} > 4$ ) it becomes

$$(32) \quad K'_a = \frac{[\text{H}^+] (B - [\text{OH}'])}{C - (B - [\text{OH}'])}$$

We may test the approximate accuracy of Equation 30 in the acid and alkaline range by applying it to the titration curves of W. M. Clark<sup>5</sup> (1920), for  $\text{H}_3\text{PO}_4 + \text{KOH}$  and for  $\text{H}(\text{K}_2\text{PO}_4) + \text{KOH}$ . (For the neutral range it has already been tested in the form of Henderson's equation in Table I.) Clark titrated 50 cc. of 0.1 M (or 0.3 N)  $\text{H}_3\text{PO}_4$  with 150 cc. of 0.1 N  $\text{KOH}$ , and determined the pH curve. The volume was, therefore, increasing throughout the titration. From Clark's curves we obtain the data of Table III. The published curves are drawn on a scale which limits pH readings to an accuracy of about 0.05, but this suffices, considering the wide range covered, to permit use of the data to test the approximate accuracy of Equation 30. As seen from the last 2 columns, the equation gives a true constant.

In order to differentiate Equation 30 we substitute  $\frac{K_w}{[\text{H}^+]}$  for  $[\text{OH}']$ , and  $K_w$  for the term  $[\text{H}^+] [\text{OH}']$ , solve the equation for B, and obtain

$$\begin{aligned} B &= \frac{K'_a C [\text{H}^+] - K'_a [\text{H}^+]^2 - [\text{H}^+]^3 + K_w K'_a + K_w [\text{H}^+]}{[\text{H}^+] (K'_a + [\text{H}^+])} \\ &= \frac{[\text{H}^+] K'_a C}{[\text{H}^+] (K'_a + [\text{H}^+])} - \frac{[\text{H}^+]^2 (K'_a + [\text{H}^+])}{[\text{H}^+] (K'_a + [\text{H}^+])} + \frac{K_w (K'_a + [\text{H}^+])}{[\text{H}^+] (K'_a + [\text{H}^+])} \\ (33) \quad &= \frac{K'_a C}{K'_a + [\text{H}^+]} - [\text{H}^+] + \frac{K_w}{[\text{H}^+]} \end{aligned}$$

$$\begin{aligned} (34) \quad \frac{dB}{d[\text{H}^+]} &= - \frac{K'_a C}{(K'_a + [\text{H}^+])^2} - 1 - \frac{K_w}{[\text{H}^+]^2} \\ \frac{dB}{dpH} &= - 2.3 [\text{H}^+] \frac{dB}{d[\text{H}^+]} \end{aligned}$$

$$(35) \quad \beta = \frac{dB}{dpH} = 2.3 \left( \frac{K'_a C [\text{H}^+]}{(K'_a + [\text{H}^+])^2} + [\text{H}^+] + [\text{OH}'] \right)$$

<sup>5</sup> Clark (1920), p. 32.

TABLE III.  
*Calculation of  $K'_a$  Values of  $H_3PO_4 - KH_2PO_4$  and of  $HK_2PO_4 - K_3PO_4$  Mixtures by Means of Equation 30.*

Mixtures present.	0.1 N KOH added to 50 cc. 0.1 M $H_3PO_4$ .	V total volume of solution.	C $= \frac{0.1 \times 50}{V}$	B $= \frac{0.1 \times \text{cc. KOH}}{V}$	pH	$[H^+]$	$[OH^-]$	$K'_a$	$pK'_a =$ $-\log K'_a$
	cc.	cc.	N	N		N	N		
$H_3PO_4 -$ $KH_2PO_4$	0	50	0.1000	0.0000	1.60	0.0251	0.0000	$0.84 \times 10^{-2}$ *	2.08
	10	60	0.0834	0.0167	1.80	0.0159	0.0000	1.01 "	2.00
	20	70	0.0714	0.0286	2.00	0.0100	0.0000	1.18 "	1.93
	30	80	0.0625	0.0375	2.30	0.0050	0.0000	1.06 "	1.97
	40	90	0.0556	0.0444	2.70	0.0020	0.0000	1.01 "	2.00
Average of last 4 calculations.....									
$HK_2PO_4 -$ $K_3PO_4$	100 + 10	160	0.0313†	0.0062	11.20	$6.31 \times 10^{-2}$	0.0016	$1.10 \times 10^{-2}$	11.96
	100 + 20	170	0.0294	0.0118	11.55	2.82 "	0.0036	1.08 "	11.97
	100 + 30	180	0.0278	0.0168	11.80	1.59 "	0.0063	0.96 "	12.02
	100 + 40	190	0.0263	0.0211	11.95	1.12 "	0.0089	0.97 "	12.01
Average.....									
									$1.03 \times 10^{-2}$

\* The first figures,  $0.84 \times 10^{-2}$  and 2.08, really represent  $K_a$  rather than  $K'_a$ , since there is present only free acid without salt. Since  $K'_a = \gamma_s K_a$ , if  $\gamma_s$  in the other determinations has a value of 0.8, they would yield an average  $K'_a$  value of  $0.8 \times 1.08 \times 10^{-2} = 0.86 \times 10^{-2}$ .

† The base,  $[B]$ , is calculated from the amount added in excess of the 100 cc. required to neutralize the first two hydrogens of  $H_3PO_4$ .

Equation 35 is identical with Equation 17, which expresses the entire range of buffer values of a solution containing a weak acid plus amounts of strong acid or alkali not limited to equivalence with the weak buffer acid. It is evident therefore, that Equation 35, as an approximation, is valid in general for solutions containing acid buffers of all dissociation constants, at all pH's and concentration ranges, within which the mass action law holds—that is, for buffer concentrations up to about 0.1 M, and a pH range between 2 and 12, and perhaps as wide as between 1 and 13, and for amounts of added strong alkali or acid not limited to any relationship to the amount of buffer acid present.

It is evident from Equation 35 that when  $[H^+]$  or  $[OH']$  is of significant magnitude in comparison with  $\frac{K'_a C}{(K'_a + [H^+])^2}$ , the buffer effect will be correspondingly greater than that calculated from Equation 12 on the basis of Henderson's equation. Such is the case with the higher acidities attainable with  $H_3PO_4 - BH_2PO_4$  mixtures, and with the higher alkalinities attainable with  $HB_2PO_4 - B_3PO_4$  mixtures. Thus in Fig. 6 we have in the central part, the curve of  $H(KHPO_4) - K(KHPO_4)$  mixtures, typical in shape and symmetry for a buffer acid acting in conformity with Henderson's equation, and with its center at  $pH = pK'_a$ ; while the other two ends curve symmetrically towards horizontal asymptotes. The  $H_3PO_4 - KH_2PO_4$  curve, however, instead of showing a decreasing slope as the proportion of free  $H_3PO_4$  exceeds  $\frac{1}{2}$  molecule, shows a slope constantly increasing to the end. This is because, as shown in Equation 35, the buffer effect of the  $[H^+]$  itself, represented by the separate term  $[H^+]$  in the equation 
$$\beta = 2.3 \left( \frac{K'_a C [H^+]}{K'_a + [H^+]} + [H^+] + [OH'] \right),$$
 becomes so great, at the low pH (1.5) reached, that it prevents the decrease in the slope of the pH curve that otherwise occurs as  $[H_2A]$  approaches C, and in fact causes an increase. A similar increase of slope is observed, because of the high  $[OH']$ , at the alkaline end of the  $HK_2PO_4 - K_3PO_4$  curve.

The quantitative rôles are indicated by Fig. 7 of the buffer effects due to incomplete dissociation of the phosphoric acids, and the buffer effects due to the  $[H^+]$  and  $[OH']$ , respectively, at the

more extreme pH limits by the two lower curves in somewhat lighter lines than the upper curve representing the total effect. The curves are calculated by Equation 35 using  $K'_a$  values of 1.97, 6.85, and 12.0, respectively, for the three acids.

It will be noted that the  $\beta$  values at the alkaline end of the curve in Fig. 7 are somewhat less than indicated by the slope of this end of the curve in Fig. 6. The disagreement is not actual, however. Fig. 7 is calculated on the assumption of a constant volume with  $C = 0.1$  M; while Fig. 6 represents the results of a titration in which the volume was greatly increased at the end. The disagreement would entirely disappear if the values of Fig. 7 were estimated on the basis of actual  $C$  and  $B$  values calculated from the varying volume.

*Polyvalent Acid Buffers and Mixtures of Monovalent Acid Buffers.*

For a solution containing a mixture of buffers, the buffer effect at any pH is the sum of the separate effects of each buffer in repressing  $[H^+]$  change, plus the buffer effect peculiar to the  $[H^+]$  and  $[OH']$ .

$$(36) \quad \beta = 2.3 [H^+] \left( \frac{K'_{a_1} C_1}{(K'_{a_1} + [H^+])^2} + \frac{K'_{a_2} C_2}{(K'_{a_2} + [H^+])^2} + \dots \right) + 2.3 ([H^+] + [OH'])$$

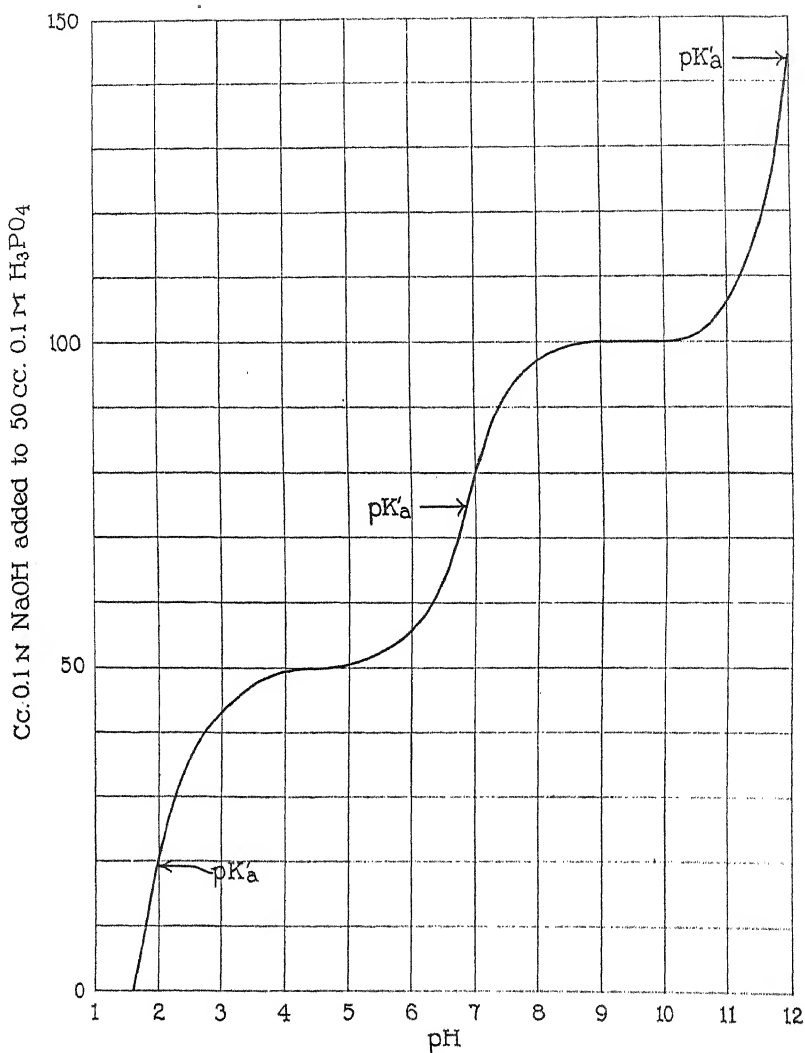
If  $C_1 = C_2 = C_3$ , as when the various acid groups belong to the same molecule of a polyvalent acid, Equation 36 simplifies to

$$(37) \quad \beta = 2.3 [H^+] C \left( \frac{K'_{a_1}}{(K'_{a_1} + [H^+])^2} + \frac{K'_{a_2}}{(K'_{a_2} + [H^+])^2} + \dots \right) + 2.3 ([H^+] + [OH'])$$

In a polyvalent buffer, or a mixture of buffers, the  $K'_a$  value and buffer effect  $\beta_1$ ,  $\beta_2$ , or  $\beta_3$ , etc., of any one acid or acid group may be determined, under the following conditions.

1. *The  $pK'_a$  values of the different acids or acid groups are, as in  $H_3PO_4$ , so far apart that the buffer effects do not overlap.* In this case at  $[H^+]$  values where  $\beta_1$  is of significant magnitude,  $\beta_2$  and  $\beta_3$  are not, and  $\beta = \beta_1$ ,  $\beta_2$ , and  $\beta_3$  being practically zero.

The extent to which a buffer exerts its effect on both sides of the point  $pH = pK'_a$  is roughly indicated by the following table,

FIG. 6. Clark's titration curve of  $\text{H}_3\text{PO}_4$ .

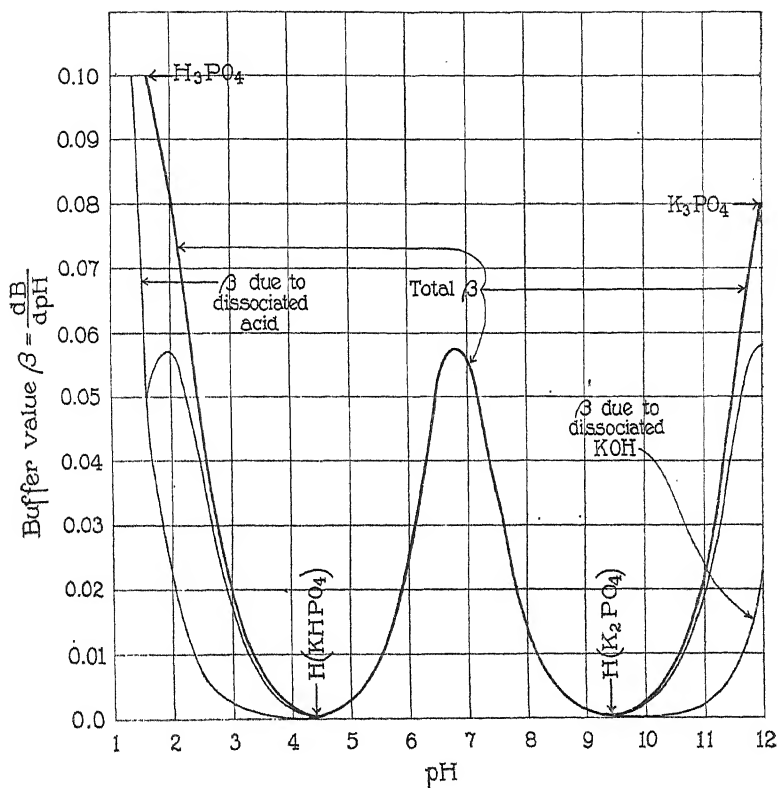


FIG. 7. Buffer values of the three phosphoric acids. The total buffer value is indicated by the heavy upper curve, which represents the total  $\beta$  value calculated by Equation 35.

At the more acid and alkaline ranges the partial  $\beta$  values due solely to dissociated free acid and alkali, respectively, become significant, and are indicated by the marked lower curves; while the remaining partial  $\beta$  values, due to repression of  $[H^+]$  change, are indicated by symmetrical curves similar to that of  $H(BHPO_4)$  in the center.

pH	Proportion of maximal buffer effect.
	<i>per cent</i>
$pK'_a$	100.0
$pK'_a \pm 1$	33.0
$pK'_a \pm 2$	4.0
$pK'_a \pm 3$	0.4
$pK'_a \pm 4$	0.04

(The effect is accurately shown for all values within the range  $pH = pK'_a \pm 2$  by Fig. 9 in the attached note by Henderson.)

An example of a polyvalent buffer with acid groups whose buffer effects do not overlap is given, as stated above, by  $H_3PO_4$ , and shown in Figs. 5 and 6.

2. *The buffer effects partially overlap.* In this case the calculations can be made from the parts of the curve where each buffer is free from the influence of the other, and can be checked by calculations of the total  $\beta$  and  $B$  in the pH range where they are summations of the overlapping effects.

3. *The effective range of a buffer is completely overlapped by those of others, of which, however, the  $K'_a$  and  $C$  values are known.* In this case we may assume for example that two buffers overlap with their buffer effects  $\beta_1$  and  $\beta_2$  the effect  $\beta_3$  of a third buffer. If  $\beta_1$  and  $\beta_2$  can be calculated,  $\beta_3$  can be estimated by subtracting  $\beta_1$  and  $\beta_2$  from the total observed buffer value,

$$\beta_3 = \beta - \beta_1 - \beta_2$$

From  $\beta_3$  thus ascertained one may calculate  $K'_a$  by Equation 22. The calculation of  $\beta_1$  and  $\beta_2$  from known  $K'_{a_1}$  and  $K'_{a_2}$ , and of  $K'_{a_3}$  from  $\beta_3$ , is made most simply, however, graphically, by inspection of the U-curve of Fig. 9.

When the total amount of base,  $B$ , is known, a similar procedure can be followed to use it instead of  $\beta$  to determine the dissociation constants. From Equation 33 one determines  $B_1$  and  $B_2$ , and by subtracting them from the total base  $B$  obtains  $B_3$ . This is substituted in Equation 30 to determine  $K'_{a_3}$ . This procedure is somewhat simpler than that based on  $\beta$  determinations.

An example of the use of the above modes of procedure in determining the separate dissociation constants of a polyvalent acid is contained in a subsequent paper by Hastings and Van Slyke (1922) on citric acid.

It is to be noted that the maximum  $\beta_M$  of a polyvalent buffer may exceed 0.575 if the effects of the different buffer groups overlap. When  $\beta = \beta_1 + \beta_2 + \beta_3$ , if at the pH where  $\beta_2$  has its maximum value of 0.575,  $\beta_1$  and  $\beta_3$  are of significant size, we shall have  $\beta = 0.575 + \beta_1 + \beta_3$ . In the case of citric acid, for example, the maximum  $\beta_M$  is 0.84. See also Fig. 8 for demonstration of the summation of buffer effects when the  $pK'$  values are nearer together than 3 units.

### *Basic Buffers.*

If we designate the undissociated weak base as bOH (a small b is used to differentiate the weak base from B, the strong base), we have as our primary equation for dissociation of a weak base, Equation 38, analogous to Equation 5.

$$(38) \quad K_b = \frac{[\text{OH}'] [\text{b}^+]}{\text{bOH}}$$

If we have a mixture of a salt bA and the free base bOH, the equation becomes, analogous to Equation 28

$$(39) \quad K_b = \frac{[\text{OH}'] \left( \gamma_s A - \frac{\gamma_s}{\gamma_A} [\text{H}^+] + [\text{OH}'] \right)}{C - A + \frac{[\text{H}^+]}{\gamma_s} - [\text{OH}']}$$

If we let  $K'_b = \frac{K_w \gamma_s}{K_b}$ , substitute in Equation 39  $\frac{K_w \gamma_s}{K_b}$  for  $K_b$ ,  $\frac{K_w}{[\text{H}^+]}$  for  $[\text{OH}']$ , and  $-B$  for  $A$ , and differentiate  $B$  with respect to  $[\text{H}^+]$ , as in obtaining Equation 35, we obtain an equation (Equation 40) identical with Equation 35, except that  $K'_b$  replaces  $K'_a$ .

$$(40) \quad \frac{dB}{dpH} = 2.3 \left( \frac{K'_b C [\text{H}^+]}{(K'_b + [\text{H}^+])^2} + [\text{H}^+] + [\text{OH}'] \right)$$



It follows, therefore, that all the equations and relationships that have been demonstrated to hold for acid buffers hold likewise for basic ones, the only difference being that in equations covering the behavior of the latter the approximate constant  $K'_b$ , which equals  $\frac{K_w \gamma_s}{K_b}$ , replaces the approximate constant  $K'_a$  which equals  $\frac{K_a}{\gamma_s}$ , of the equations covering the behavior of acid buffers.

### *Amphoteric Buffers.*

In the case of an amphoteric buffer with acid groups, having  $K'_a$  values  $K'_{a_1}$ ,  $K'_{a_2}$ , etc., and basic groups having  $K'_b$  values  $K'_{b_1}$ ,  $K'_{b_2}$ , etc., we have, by summation

$$(41) \quad \beta = 2.3 C [H^+] \left( \frac{K'_{a_1}}{(K'_{a_1} + [H^+])^2} + \frac{K'_{a_2}}{(K'_{a_2} + [H^+])^2} \dots + \frac{K'_{b_1}}{(K_w + K'_{b_1} [H^+])^2} + \frac{K'_{b_2}}{(K_w + K'_{b_2} [H^+])^2} \right) + 2.3 ([H^+] + [OH'])$$

The discussion under "Polyvalent acid buffers" applies also to amphoteric buffers, practically without modification except that  $pK'_b$  is to be used in place of  $pK'_a$  for the basic group.

### *Universal Buffer Mixtures.*

Acree and his coworkers (1921) have pointed out that "there are advantages to be derived from the use of buffer mixtures covering a wide pH range . . . and so selected as to form a practically continuous, as well as smooth curve," in other words, to maintain a constant buffer value. From the considerations of the present paper it appears possible to predict the relationships of the buffers that will form such a mixture. Approximate constancy of buffer value will be maintained in a mixture of total buffer value

$$\beta = \beta_1 + \beta_2 + \beta_3 \dots$$

under the following conditions. Each buffer must have its  $pK'$  at such a distance from its adjacent neighbor in the series that

the overlapping buffer effects at  $\text{pH} = \text{pK}'_1$  equal those at  $\text{pH} = \frac{\text{pK}'_1 + \text{pK}'_2}{2}$ , or at a pH midway between two  $\text{pK}'$  values. Under

these conditions the total buffer value midway between the maximum of a given buffer and that of the adjacent buffer will be the same as the buffer values at the maxima, and the fluctuations between can be but slight. The solution of the problem is approximated by calculating the total buffer values by means of Equation 12. The calculation is most readily done graphically by means of Fig. 9. Over most of the range covered in the calculations of Table II, it was necessary to add 5 buffer values at the point  $\text{pH} = \text{pK}'_1$ , and 6 at the point  $\text{pH} = \frac{\text{pK}'_1 + \text{pK}'_2}{2}$ , midway between 2  $\text{pK}'$ 's.

TABLE IV.

Interval between $\text{pK}'_1$ and $\text{pK}'_2 \dots \text{etc.}$	Total buffer value, calculated as sum of molecular buffer values of individual buffers.	
	at $\text{pH} = \text{pK}'$	at $\text{pH} = \text{pK}' + \frac{i}{2}$
$i$		
3.0	0.577	0.138
2.0	0.598	0.384
1.6	0.634	0.552
1.4	0.749	0.673
1.3	0.784	0.738
1.2	0.848	0.813
1.1	0.919	0.899
1.0	1.003	0.998

It is apparent from Table IV that the ideal universal buffer would be approximated by a mixture of buffers forming a series with their  $\text{pK}'$  values separated by 1 unit, each  $\text{K}'$  being 10-fold as great as the next lower. In a solution containing such a series of buffers in equivalent concentration the total buffer value varies less than 1 per cent. When the intervals between  $\text{pK}'$ 's are 1.4, the total buffer value shows a difference of about 10 per cent between maxima and minima. When the difference is 2.0 the maximum buffer values exceed the minimum by over 50 per cent.

When the  $\text{pK}'$  intervals are regular, the most constant total buffer value is obtainable by using all buffers in the same concen-

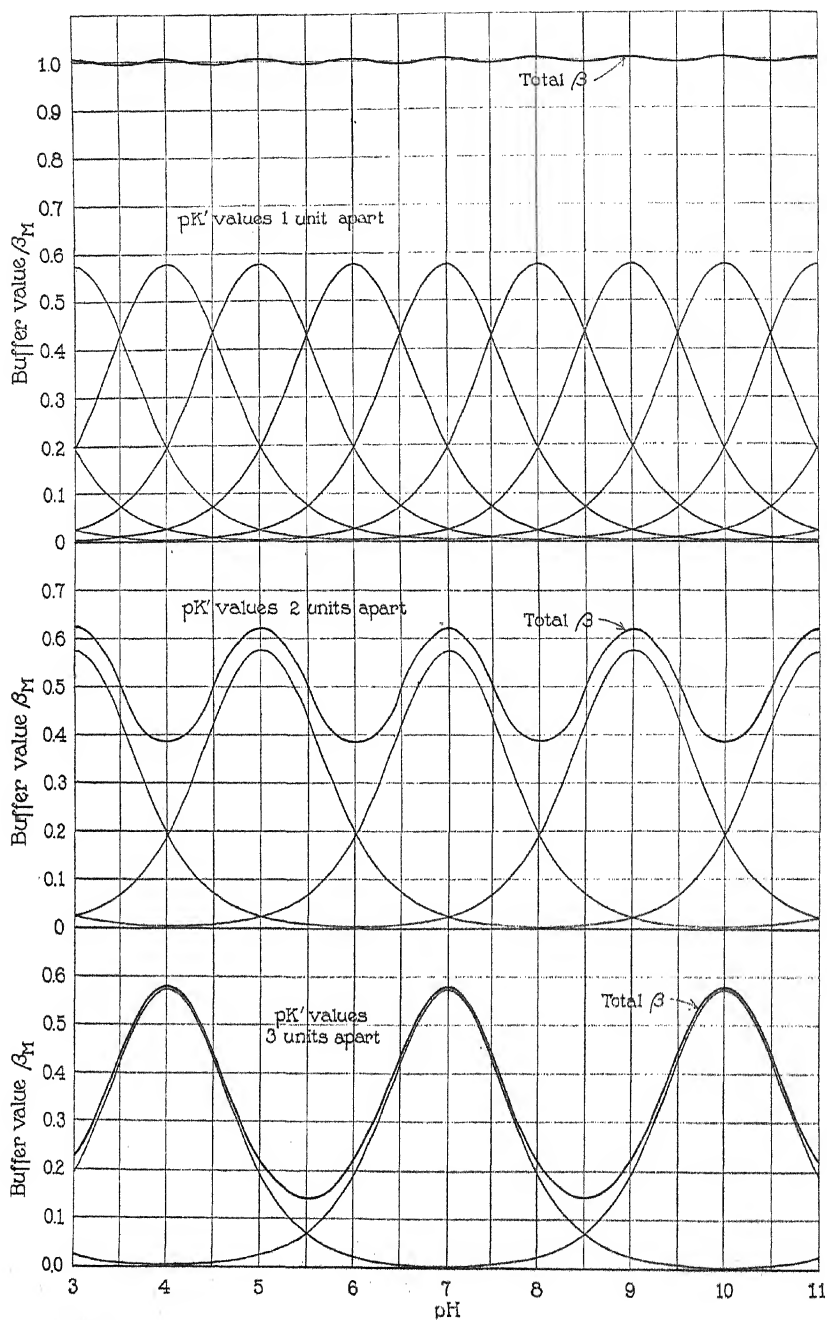


FIG. 8.

tration. In case, however, some of the intervals are longer than the rest so that one or two buffers are more isolated from the rest and consequently less supported by overlapping buffer effects of their neighbors, such buffers are to be used in sufficiently greater concentration to bring the total buffer value in these zones of activity up to that of the rest of the system.

Fig. 8 shows graphically the total buffer values obtained by equimolecular mixtures of buffer series, of which the  $pK'$  intervals are 1, 2, and 3, respectively.

### *The Buffer Value of Blood.*

From the  $BHCO_3$  : pH curve for normal arterial blood under varying  $CO_2$  tensions given by the writer in a previous article (1921, *b*, Fig. 2) it is possible to calculate the buffer value. At the reaction range pH 7.0 to 7.8,  $dB = dBa$ , since the amount of B in the form of BOH is negligible. The increase in  $BHCO_3$  with increasing  $CO_2$  tension and falling pH is equal to the decrease in Ba of the other buffers, since the base used to form additional  $BHCO_3$  comes from them. Consequently, for the buffers other than  $BHCO_3$ ,  $\frac{dB}{dpH} = \frac{-dBHCO_3}{dpH}$ . Between pH 7.0 and 7.8 (mean pH = 7.4;  $\Delta pH = 0.8$ ) the mean molecular concentration of  $BHCO_3$  decreases from 0.0285 to 0.0120 ( $\Delta Ba = 0.0165$ ). Therefore, at mean pH 7.4, for the buffers other than  $BHCO_3$ ,  $\beta = \frac{\Delta Ba}{\Delta pH} = \frac{0.0165}{0.8} = 0.0206$ .

The buffers other than  $BHCO_3$  are chiefly proteins. In fact, they appear to be chiefly the weak acid groups in the hemoglobin (Van Slyke, 1921, *a*).<sup>6</sup> If these groups were exerting their maximum buffer efficiency, having their  $pK'$  values near 7.4, their total concentration would be  $C = \frac{\beta}{0.575} = \frac{0.0206}{0.575} = 0.036$  N (Equation 20), and they would be combined with 0.5 equivalent of alkali, or 0.018 N, since, at  $pH = pK'$ ,  $Ba = \frac{C}{2}$ . If  $pK'$  were less than 7.4, then Ba would exceed 0.018 N, if  $pK'$  were more than 7.4, then Ba, the base bound to protein, would be less than 0.018 N.

<sup>6</sup> Van Slyke (1921, *a*), p. 160.

As a matter of fact, as closely as one can estimate from the only data available (Greenwald, 1922), 0.018  $N$  is about the concentration of alkali bound to proteins in the blood. If this is substantiated (experiments are in progress), it means that in normal blood the active buffer groups, other than those of  $\text{BHCO}_3$ , total about 0.036  $N$  in concentration. The hemoglobin concentration is about 0.008  $M$ , if one assumes that 1 molecule of hemoglobin binds 1 molecule of oxygen. Probably, therefore,  $\frac{3.6}{0.8} = 4.5$  represents more or less approximately the number of  $\text{COOH}$  groups in each oxyhemoglobin molecule that are active at blood pH.

In Greenwald's method the proteins are precipitated with an excess of picric acid and the picric acid combined with alkali is determined as the difference between total picrate and free titratable picric acid. Essentially this procedure removes the protein and total  $\text{H}_2\text{CO}_3$ , and measures the picric acid required to neutralize the bases that were combined with them. In order to measure this value exactly in such a procedure the titration of the free picric acid would require the pH of blood for its end-point (Van Slyke, Stillman, and Cullen, 1919).<sup>7</sup> Greenwald used two end-points, one (methyl red) 2 pH units on the acid side of 7.4, the other (thymolphthalein) about equally on the alkaline side. The average normal concentrations of base thus measured were 0.0435 and 0.0315  $N$ , respectively, with the two indicators, the mean value, 0.0375  $N$  being probably close to the amount of base bound by  $\text{H}_2\text{CO}_3$  and protein in normal blood. If we subtract from 0.0375  $N$  the 0.0205  $N$  base normally bound as bicarbonate (Van Slyke, 1921, *b*),<sup>8</sup> we have left 0.0170  $N$  base bound by the proteins.

If to the buffer value of the buffers other than the bicarbonate we add that of bicarbonate we obtain the total buffer value of the blood. The value for the  $\text{BHCO}_3$  and  $\text{H}_2\text{CO}_3$  buffer at pH 7.4 may be calculated from Equation 12. At pH 7.4,  $[\text{H}^+] = 4 \times 10^{-8}$ ;  $C = [\text{BHCO}_3] + [\text{H}_2\text{CO}_3] = 0.0215$ ; and  $K'_a = 8 \times 10^{-7}$  (Henderson and Haggard). Substituting these numerical values in Equation 12 we have

$$\frac{dB}{d\text{pH}} = 2.3 \frac{4 \times 10^{-8} \times 0.0215 \times 8 \times 10^{-7}}{(8 \times 10^{-7} + 4 \times 10^{-8})^2} = 0.0022$$

Adding 0.0022 to 0.0206, the  $\beta$  of the other buffers, gives  $\beta = 0.0228$  as the average total buffer value of normal human blood at pH 7.4.

<sup>7</sup> Van Slyke, Stillman, and Cullen (1919), p. 167.

<sup>8</sup> Van Slyke (1921, *b*), p. 170.

The total *concentration* of buffers active in the blood at pH 7.4 is at least 0.036 N (buffers other than bicarbonate) + 0.021 N ( $\text{BHCO}_3 + \text{H}_2\text{CO}_3$ ) = 0.057 N. The alkali bound by these buffers, which are apparently all of the weak acid type, appears to be about 0.04 N (0.038 N bound by protein and  $\text{H}_2\text{CO}_3$  (Greenwald, 1922), and traces by other buffers).

The above estimates of buffer concentration and buffer alkali are merely first approximations. The 0.04 N buffer alkali figure is a rough estimate. The 0.057 N total buffer concentration may have to be increased in proportion as the active protein buffer groups are found to have  $\text{pK}'$  values differing from 7.4. The figure 0.022 for the average  $\frac{dB}{dpH}$  value of human blood is based on fairly complete observations and is probably about correct.

The ability of the blood *in vivo* to neutralize *non-volatile acids without change in pH* is not due to buffer action, that is to the incomplete dissociation of the weak buffer acids set free by reactions such as  $\text{HCl} + \text{Ba} = \text{Ha} + \text{BCl}$ . It is due to the volatility of one of these acids,  $\text{CO}_2$ , and the ability of the respiratory apparatus to remove the excess  $\text{CO}_2$  set free by such a reaction as  $\text{HCl} + \text{BHCO}_3 = \text{BCl} + \text{H}_2\text{CO}_3$ . Because  $\text{CO}_2$  set free is thus removed, the bicarbonate is, as shown by the writer (1921, a), the most important form of the blood's alkali reserve available for neutralizing non-volatile acids, such as  $\beta$ -hydroxybutyric.

For neutralization of added  $\text{H}_2\text{CO}_3$ , however, (in venous blood) the  $\text{BHCO}_3$  formed can exert only its actual buffer effect, which is about 0.1 that of the other blood buffers. For preservation of the blood's neutrality against retention of non-volatile acids the bicarbonate, because of the volatility of the  $\text{CO}_2$  set free from it, is the most important single constituent; while for preservation of neutrality against  $\text{CO}_2$  retention, hemoglobin, because of its buffer power, is the most important constituent.

#### *Determination of the Buffer Values of a Solution by Titration.*

The experimental determination of the buffer value,  $\beta$ , of a given solution at a given pH may be performed in either of the following two ways.

1. *By determination of the slope of the B, pH curve at the given pH.* One adds strong standard acid or alkali in varying amounts

to the solution, and determines the pH after each addition. If the concentration of added standard acid or alkali be great enough to keep the maximum increase in volume of the buffer solution relatively small, *e.g.* below 50 per cent of the original, the volume change may ordinarily be neglected, as the pH values of most buffer solutions are but slightly affected by volume changes of such magnitude. Addition of each cc. of *N* NaOH to a buffer solution of original volume *V* cc. causes an increase of  $\Delta B = \frac{1}{V}$ .

The buffer value,  $\frac{dB}{dpH}$ , at any pH may be determined graphically by drawing a line tangent to the curve at that pH and measuring the slope of the line. (See Fig. 1 and its discussion.) (The tangent can be drawn most accurately by a device employed by engineers and called to my attention by Dr. Northrop. A mirror is laid at right angles across the curve, so that the curve on paper is continuous with its reflection. A line drawn across the curve along the edge of the mirror is then perpendicular to the tangent, which with the help of a square is laid off at right angles to the mark of the mirror-edge.)

2. *By measuring the amount of strong acid or alkali required to cause a pH change over a measured range.* One merely adds as in the above procedure, a measured amount of standard alkali or acid, and determines the pH before and after the addition. If the added alkali is  $\Delta B$ , and the increase in pH is  $\Delta pH$ , then the

average buffer value between the two pH's observed is  $\beta = \frac{\Delta B}{\Delta pH}$ .

For example, if 10 cc. of *M* NaOH are added to 1 liter of a buffer solution of unknown value,  $\Delta B = \frac{10 \times 1}{1,000} = 0.01$ . If the pH values before and after the additions are 7 and 8.5, respectively  $\Delta pH = 8.5 - 7 = 1.5$ ,  $\beta = \frac{0.01}{1.5} = 0.0067$ . 0.0067 represents the average value of  $\beta$  between pH 7 and 8.5, or approximately the  $\beta$  at pH 7.75. Table I gives an example of this manner of determining values of  $\beta$ .

If the average  $\beta$  value over a certain pH range is used as an approximation of the exact  $\beta$  value at the mean pH, as in Table

I, it is essential that the pH intervals be not too great. Where the B, pH curve is nearly straight a pH range exceeding 1 may be used to determine  $\beta$  at the mean pH, as in the case of citric acid; but where there is much curvature the range should be less in order to yield accurate mean values.

TABLE V.

$\frac{[\text{Ba}]}{C} \text{ or } \frac{\text{bOH}}{C}$	$\frac{[\text{Ha}]}{C} \text{ or } \frac{\text{bA}}{C}$	$\frac{[\text{H}^+]}{K}$	$\log \frac{[\text{H}^+]}{K} = \text{pK}' - \text{pH}$	$0.4343 \beta_M = V$	$\frac{dV}{d \log [\text{H}^+]}$	$\frac{\beta}{0.575} = U$
0.01	0.99	99.0	1.996	0.0099	-0.0097	0.0396
0.02	0.98	49.0	1.690	0.0196	-0.0192	0.0784
0.05	0.95	19.0	1.279	0.0475	-0.0428	0.1900
0.10	0.90	9.0	0.954	0.0900	-0.0720	0.3600
0.15	0.85	5.67	0.753	0.1275	-0.0893	0.5100
0.20	0.80	4.00	0.602	0.1600	-0.0960	0.6400
0.25	0.75	3.00	0.477	0.1875	-0.0938	0.7500
0.30	0.70	2.33	0.368	0.2100	-0.0840	0.8400
0.35	0.65	1.858	0.269	0.2275	-0.0683	0.9100
0.40	0.60	1.500	0.176	0.2400	-0.0480	0.9600
0.45	0.55	1.222	0.087	0.2475	-0.0248	0.9900
0.50	0.50	1.000	0.000	0.2500	0.0000	1.0000
0.55	0.45	0.818	0.913-1	0.2475	0.0248	0.9900
0.60	0.40	0.667	0.824-1	0.2400	0.0480	0.9600
0.65	0.35	0.538	0.731-1	0.2275	0.0683	0.9100
0.70	0.30	0.429	0.632-1	0.2100	0.0840	0.8400
0.75	0.25	0.333	0.523-1	0.1875	0.0938	0.7500
0.80	0.20	0.250	0.398-1	0.1600	0.0960	0.6400
0.85	0.15	0.176	0.247-1	0.1275	0.0893	0.5100
0.90	0.10	0.111	0.046-1	0.0900	0.0720	0.3600
0.95	0.05	0.0526	0.721-2	0.0475	0.0428	0.1900
0.98	0.02	0.0204	0.310-2	0.0196	0.0192	0.0784
0.99	0.01	0.0101	0.004-2	0.0099	0.0097	0.0396

*Note by Lawrence J. Henderson.*

Professor Henderson consented to criticize the preliminary draft of the above paper and suggested that the relationships involved in the first and second differential derivatives of his equation (Equation 7) might be more readily perceived as out-



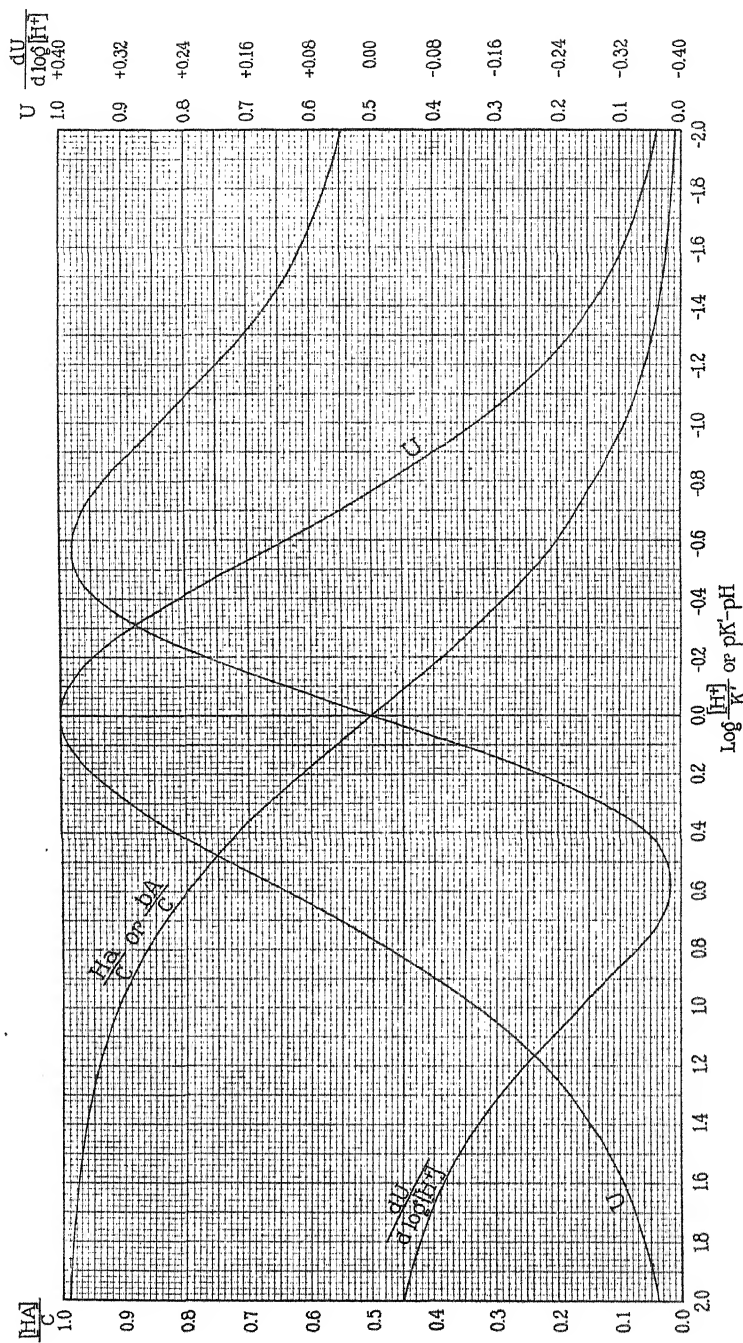


FIG. 9.

lined by the following series of equations, and by Table V and Fig. 9. The latter demonstrate over the main significant range, the interrelation between: (1) buffer effect, (2) ratio of buffer dissociation constant to hydron concentration of the solution, (3) ratio of buffer salt to free buffer base or acid, and (4) value of the second differential.

*First Derivative.*

Given a buffer mixture such that

$$[\text{Ha}] + [\text{Ba}] = C$$

If 
$$[\text{H}^+] = K \frac{[\text{Ha}]}{[\text{Ba}]}$$

$$[\text{H}^+] = K \frac{[\text{Ha}]}{C - [\text{Ha}]}$$

$$[\text{H}^+] C = [\text{H}^+] [\text{Ha}] + K [\text{Ha}]$$

$$[\text{Ha}] = \frac{[\text{H}^+] C}{K + [\text{H}^+]}$$

Imagine addition of strong acid or strong base

$$\frac{d[\text{Ha}]}{d[\text{H}^+]} = \frac{(K + [\text{H}^+]) C - [\text{H}^+] C}{(K + [\text{H}^+])^2} = \frac{C}{K + [\text{H}^+]} - \frac{C}{K + [\text{H}^+]} \times \frac{[\text{H}^+]}{K + [\text{H}^+]}$$

$$\frac{d[\text{Ha}]}{d[\text{H}^+]} = \frac{C}{K + [\text{H}^+]} \left(1 - \frac{[\text{H}^+]}{K + [\text{H}^+]}\right) = \frac{C}{K + [\text{H}^+]} \times \frac{K}{K + [\text{H}^+]}$$

$$\beta_M \times \log_e 10 = V = \frac{d[\text{Ha}]}{d[\text{H}^+]} \times \frac{d[\text{H}^+]}{d \log [\text{H}^+]} \times \frac{1}{C} = \frac{d[\text{Ha}]}{d \log [\text{H}^+]} \times \frac{1}{C} = \frac{[\text{H}^+]}{K + [\text{H}^+]} \times \frac{K}{K + [\text{H}^+]}$$

$$V = \frac{1}{1 + \frac{[\text{H}^+]}{K}} \times \frac{1}{1 + \frac{K}{[\text{H}^+]}} \dots \dots \dots (1)$$

But 
$$\frac{[\text{Ha}]}{C} = \frac{[\text{H}^+]}{K + [\text{H}^+]} = \frac{1}{1 + \frac{K}{[\text{H}^+]}}$$

And 
$$\frac{[\text{Ba}]}{C} = \frac{1}{1 + \frac{[\text{H}^+]}{K}}$$

Hence alternatively, leaving out the constant factor  $\frac{1}{\log_e 10}$ ,

$$\left\{ \begin{array}{l} \beta_M = \frac{[\text{Ha}]}{C} \times \frac{[\text{Ba}]}{C} \\ \beta_M = \frac{[\text{Ha}]}{C} - \frac{[\text{Ha}]^2}{C^2} \\ \beta_M = \frac{[\text{Ba}]}{C} - \frac{[\text{Ba}]^2}{C^2} \end{array} \right.$$

*Second Derivative.*

$$\beta_M \times \log_e 10 = V = \frac{H}{K + [H^+]} \times \frac{K}{K + [H^+]}$$

$$\frac{dV}{d[H^+]} = \frac{K}{K + [H^+]} \times \frac{(K + [H^+]) - [H^+]}{(K + [H^+])^2} + \frac{[H^+]}{K + [H^+]} \times \frac{-K}{(K + [H^+])^2}$$

$$\frac{dV}{d \log [H^+]} = \frac{dV}{d[H^+]} \times \frac{d[H^+]}{d \log [H^+]} = \frac{K}{K + [H^+]} \times \frac{K}{K + [H^+]} \times \frac{[H^+]}{K + [H^+]} -$$

$$\frac{K}{K + [H^+]} \times \frac{[H^+]}{K + [H^+]} \times \frac{[H^+]}{K + [H^+]}$$

$$\frac{dV}{d \log [H^+]} = V \left( \frac{1}{1 + \frac{[H^+]}{K}} - \frac{1}{1 + \frac{K}{[H^+]}} \right) \dots \dots \dots (2)$$

Hence, alternatively, leaving out the constant factor  $\frac{1}{(\log_e 10)^2}$

$$\frac{dV}{d \log [H^+]} = \frac{[Ha]}{C} \times \frac{[Ba]}{C} \times \left( \frac{[Ba]}{C} - \frac{[Ha]}{C} \right)$$

## SUMMARY.

As a numerical measure of the buffer value of a solution the number of gram equivalents of strong alkali or acid taken up per unit change in pH has been used. In these terms, each cc. of  $N$  alkali or acid that must be added to a liter of solution to raise or lower its pH by 1 adds 0.001 to the buffer value.

Since the buffer value varies with varying pH, the value at any given pH is defined by the ratio  $\frac{dB}{dpH}$ .

A general form of the mass action equations,  $K = \frac{[H^+][a']}{[Ha]}$

and  $K = \frac{[OH'] [b']}{[bOH]}$ , covering the dissociations of weak acids and bases, respectively, in the presence of their salts, has been developed and differentiated. The general equation obtained expressing the buffer value,  $\beta$ , is

$$\beta = \frac{dB}{dpH} = 2.3 \left( \frac{K' [H^+] C}{(K' + [H^+])^2} + [H^+] + [OH'] \right)$$

$K' = \frac{K_a}{\gamma_s}$  for weak acids, where  $K_a$  is the acid dissociation con-

stant,  $\gamma_s$  the fraction of the salt Ba dissociated into  $[B^+]$  and  $[a']$  ions. For weak bases  $K' = \frac{K_w \gamma_s}{K_b}$ . C indicates the concentration of the buffer. When  $C = 0$ , and no weak acid or base is present, the buffer equation simplifies to  $\beta = 2.3 ([H^+] + [OH'])$ , which indicates the buffer value of water plus strong acid or alkali.

When pH is within the limits of about 3 and 11, and C has a value not much less than 0.1 N,  $[H^+]$  and  $[OH']$  are relatively so small that the buffer equation simplifies to

$$\beta = 2.3 \frac{K' [H^+] C}{(K' + [H^+])^2}$$

Under these conditions the molecular buffer value  $\beta_M = \frac{\beta}{C} = 2.3 \frac{K' [H^+]}{(K' + [H^+])^2}$ .  $\beta$  has its maximum value for every buffer when  $[H^+] = K'$ . At this point  $\beta_M = \frac{2.3}{4} = 0.575$  for every buffer, and one-half the buffer is in the form of free acid or base, one-half in the form of its salt. That is, maximum buffer value is exerted when  $\frac{[H^+]}{K'} = \frac{Ba}{Ha} \left( \text{or} = \frac{bOH}{bA} \right) = 1$ . For given values of these ratios greater or less than 1 definite proportions of the maximum buffer effect are exerted (Fig. 9).

Applications of the above facts are indicated, including the determination of the acid and basic dissociation constants and the molecular weights of buffers, and the calculation of the buffer values of solutions of mixed, polyvalent, and amphoteric buffers. The technique for experimental determination of the buffer values of solutions is outlined.

The buffer value of normal blood at pH 7.4 is shown to approximate that of a 0.04 N solution of a buffer acid with  $K' = 10^{-7.4}$

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*Note Added to Proof.*—Koppel and Spiro (1914), in a paper overlooked until the above went to press, have utilized a similar, though not identical buffer unit, viz.

$\frac{d(S - S_0)}{dpH}$ , where  $S_0$  represents the amount of strong

acid that would produce the change  $dpH$  if added to an unbuffered solution of a given initial  $pH$ , while  $S$  represents the actual amount of strong acid required in the buffered solution to cause the same  $pH$  change. Within the  $pH$  range of validity of Henderson's equation this unit becomes numerically identical with ours (except for being its negative value), since  $S_0$  is negligible; and Koppel and Spiro have reached some conclusions identical with ours for solutions within this range, viz., concerning the occurrence of a constant, maximum, buffer value when  $K_a = [H^+]$ . For  $pH$  values outside the range of validity of Henderson's equation, Koppel and Spiro's unit is not identical with the, we believe, more simply applied  $\frac{dB}{dpH}$  unit developed in this paper.

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